

Novel method for the production of polyunsaturated fatty acids

Description

5 The present invention relates to an improved process for the specific production of poly-unsaturated ω -3 and ω -6 fatty acids and a process for the production of triglycerides having an increased content of unsaturated fatty acids, in particular ω -3 and ω -6 fatty acids having at least two double bonds and a 20 or 22 carbon atom chain length. The invention relates to the production of a transgenic organism, preferably a
10 transgenic plant or a transgenic microorganism, having an increased content of fatty acids, oils or lipids containing C₂₀- or C₂₂- fatty acids with a Δ 5, 7, 8, 10 double bond, respectively due to the expression of a Δ 8-desaturase and a Δ 9- elongase from organisms such as plants preferably Algae like *Isochrysis galbana* or *Euglena gracilis*. In addition the invention relates to a process for the production of poly unsaturated
15 fatty acids such as Eicosapentaenoic, Arachidonic, Docosapentaenoic or Docosa-hexaenoic acid through the co- expression of a Δ -8-desaturase, a Δ -9-elongase and a Δ -5 desaturase in organisms such as microorganisms or plants.

The invention additionally relates to the use of specific nucleic acid sequences
20 encoding for the aforementioned proteins with Δ -8-desaturase-, Δ -9-elongase- or Δ -5-desaturase-activity, nucleic acid constructs, vectors and organisms containing said nucleic acid sequences. The invention further relates to unsaturated fatty acids and triglycerides having an increased content of at least 1 % by weight of unsaturated fatty acids and use thereof.

25 Fatty acids and triglycerides have numerous applications in the food industry, animal nutrition, cosmetics and in the drug sector. Depending on whether they are free saturated or unsaturated fatty acids or triglycerides with an increased content of saturated or unsaturated fatty acids, they are suitable for the most varied applications;
30 thus, for example, polyunsaturated fatty acids (= PUFAs) are added to infant formula to increase its nutritional value. The various fatty acids and triglycerides are mainly obtained from microorganisms such as *Mortierella* or from oil-producing plants such as soybean, oilseed rape, sunflower and others, where they are usually obtained in the form of their triacylglycerides. Alternatively, they are obtained advantageously from
35 animals, such as fish. The free fatty acids are prepared advantageously by hydrolysis.

Whether oils with unsaturated or with saturated fatty acids are preferred depends on the intended purpose; thus, for example, lipids with unsaturated fatty acids, specifically polyunsaturated fatty acids, are preferred in human nutrition since they have a positive effect on the cholesterol level in the blood and thus on the possibility of heart disease.

5 They are used in a variety of dietetic foodstuffs or medicaments. In addition PUFAs are commonly used in food, feed and in the cosmetic industry. Poly unsaturated ω -3- and/or ω -6-fatty acids are an important part of animal feed and human food. Because of the common composition of human food poly unsaturated ω -3-fatty acids, which are an essential component of fish oil, should be added to the food to increase the

10 nutritional value of the food; thus, for example, poly unsaturated fatty acids such as Docosahexaenoic acid (= DHA, $C_{22:6}^{\Delta 4,7,10,13,16,19}$) or Eicosapentaenoic acid (= EPA, $C_{20:5}^{\Delta 5,8,11,14,17}$) are added as mentioned above to infant formula to increase its nutritional value. Whereas DHA has a positive effect of the brain development of babies. The addition of poly unsaturated ω -3-fatty acids is preferred as the addition of poly

15 unsaturated ω -6-fatty acids like Arachidonic acid (= ARA, $C_{20:4}^{\Delta 5,8,11,14}$) to common food have an undesired effect for example on rheumatic diseases such as rheumatoid arthritis. Poly unsaturated ω -3- and ω -6-fatty acids are precursor of a family of paracrine hormones called eicosanoids such as prostaglandins which are products of the metabolism of Dihomo- γ -linoleic acid, ARA or EPA. Eicosanoids are involved in the

20 regulation of lipolysis, the initiation of inflammatory responses, the regulation of blood circulation and pressure and other central functions of the body. Eicosanoids comprise prostaglandins, leukotrienes, thromboxanes, and prostacyclins. ω -3-fatty acids seem to prevent arteriosclerosis and cardiovascular diseases primarily by regulating the levels of different eicosanoids. Other Eicosanoids are the thromboxanes and leukotrienes which are products of the metabolism of ARA or EPA.

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Principally microorganisms such as *Mortierella* or oil producing plants such as soybean, rapeseed or sunflower or algae such as *Cryptocodinium* or *Phaeodactylum* are a common source for oils containing PUFAs, where they are usually obtained in the

30 form of their triacyl glycerides. Alternatively, they are obtained advantageously from animals, such as fish. The free fatty acids are prepared advantageously by hydrolysis with a strong base such as potassium or sodium hydroxide. Higher poly unsaturated fatty acids such as DHA, EPA, ARA, Dihomo- γ -linoleic acid ($C_{20:3}^{\Delta 8,11,14}$) or Docosapentaenoic acid (= DPA, $C_{22:5}^{\Delta 7,10,13,16,19}$) are not produced by oil producing plants such

as soybean, rapeseed, safflower or sunflower. A natural sources for said fatty acids are fish for example herring, salmon, sardine, redfish, eel, carp, trout, halibut, mackerel, pike-perch or tuna or algae.

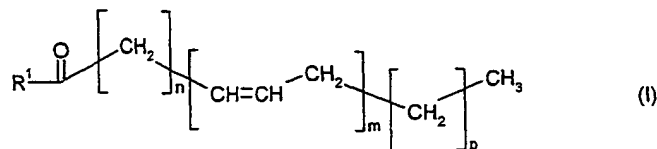
- 5 On account of their positive properties there has been no shortage of attempts in the past to make available genes which participate in the synthesis of fatty acids or triglycerides for the production of oils in various organisms having a modified content of unsaturated fatty acids. Thus, in WO 91/13972 and its US equivalent a Δ -9-desaturase is described. In WO 93/11245 a Δ -15-desaturase and in WO 94/11516
10 a Δ -12-desaturase is claimed. WO 00/34439 discloses a Δ -5- and a Δ -8-desaturase. Other desaturases are described, for example, in EP-A-0 550 162, WO 94/18337, WO 97/30582, WO 97/21340, WO 95/18222, EP-A-0 794 250, Stukey et al., J. Biol. Chem., 265, 1990: 20144-20149, Wada et al., Nature 347, 1990: 200-203 or Huang et al., Lipids 34, 1999: 649-659. To date, however, the various desaturases have
15 been only inadequately characterized biochemically since the enzymes in the form of membrane-bound proteins are isolable and characterizable only with very great difficulty (McKeon et al., Methods in Enzymol. 71, 1981: 12141-12147, Wang et al., Plant Physiol. Biochem., 26, 1988: 777-792). Generally, membrane-bound desaturases are characterized by introduction into a suitable organism which is then investigated for enzyme activity by means of analysis of starting materials and products.
20 Δ -6-Desaturases are described in WO 93/06712, US 5,614,393, US 5,614,393, WO 96/21022, WO0021557 and WO 99/27111 and their application to production in transgenic organisms is also described, e.g. in WO 9846763, WO 9846764 and WO 9846765. At the same time the expression of various fatty acid biosynthesis
25 genes, as in WO 9964616 or WO 9846776, and the formation of poly-unsaturated fatty acids is also described and claimed. With regard to the effectiveness of the expression of desaturases and their effect on the formation of polyunsaturated fatty acids it may be noted that through expression of a desaturases and elongases as described to date only low contents of poly-unsaturated fatty acids/lipids, such as by way of example
30 eicosapentaenoic or arachidonic acid, have been achieved. Therefore, an alternative and more effective pathway with higher product yield is desirable.

Accordingly, there is still a great demand for new and more suitable genes which encode enzymes which participate in the biosynthesis of unsaturated fatty acids and

make it possible to produce certain fatty acids specifically on an industrial scale without unwanted byproducts forming. In the selection of genes for biosynthesis two characteristics above all are particularly important. On the one hand, there is as ever a need for improved processes for obtaining the highest possible contents of polyunsaturated fatty acids.

Accordingly, it is an object of the present invention to provide further genes of desaturase and elongase enzymes for the synthesis of polyunsaturated fatty acids in organisms preferably in microorganisms and plants and to use them in a commercial process for the production of poly unsaturated fatty acids. Said process should increase PUFA content in organisms as much as possible preferably in seeds of an oil producing plant.

We have found that this object is achieved by a process for the production of compounds of the following general formula

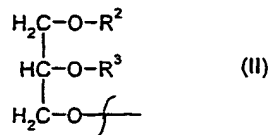


in transgenic organisms with a content of at least 1 % by weight of said compounds referred to the total lipid content of said organism which comprises the following steps:

- a) introduction of at least one nucleic acid sequence in a transgenic organism, which encodes a Δ -9-elongase, and
- b) introduction of at least one second nucleic acid sequence which encodes a Δ -8-desaturase, and
- c) if necessary introduction of at least a one third nucleic acid sequence, which encodes a Δ -5-desaturase, and
- d) cultivating and harvesting of said organism; and

where the variables and substituents in formula I have the following meanings:

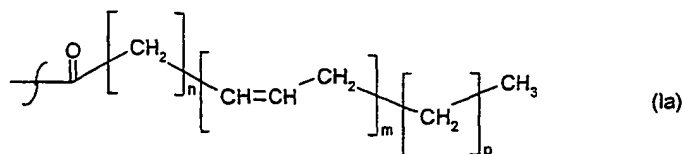
- R^1 = hydroxyl-, Coenzyme A-(Thioester), phosphatidylcholine-, phosphatidylethanol-
amine-, phosphatidylglycerol-, diphosphatidylglycerol-, phosphatidylserine-, phos-
phatidylinositol-, sphingolipid-, glycosphingolipid- or a residue of the general
formula II:



where the substituents in formula II have the following meanings:

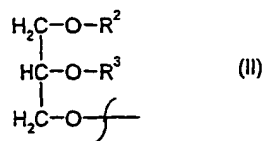
- 10 R^2 = hydrogen-, phosphatidylcholine-, phosphatidylethanolamine-, phosphatidyl-
glycerol-, diphosphatidylglycerol-, phosphatidylserine-, phosphatidylinositol-,
sphingolipid-, glycosphingolipid-, glycosphingolipid- or saturated or unsaturated
 C_2 - C_{24} -alkylcarbonyl-,
- 15 R^3 = hydrogen-, saturated or unsaturated C_2 - C_{24} -alkylcarbonyl-, or

R^2 and R^3 independent of each other a residue of the formula Ia:



- $n = 3, 4$ or 6 , $m = 3, 4$ or 5 and $p = 0$ or 3 , preferably $n = 3$, $m = 4$ or 5 and $p = 0$
or 3 .

R¹ indicates in the formula I hydroxyl-, Acetyl-Coenzyme A-, phosphatidylcholine-, phosphatidylethanolamine-, phosphatidylglycerol-, diphosphatidylglycerol-, phosphatidylserine-, phosphatidylinositol-, sphingolipid-, glycosphingolipid- or a residue of the general formula II



The abovementioned residues for R¹ are always coupled to compounds of the general formula I in the form of their ester or thioester.

R² indicates in structures of the general formula II hydrogen, phosphatidylcholine-, phosphatidylethanolamine-, phosphatidylglycerol-, diphosphatidylglycerol-, phosphatidylserine-, phosphatidylinositol-, sphingolipid-, glycosphingolipid-, glycosphingolipid- or saturated or unsaturated C₂-C₂₄-alkylcarbonyl-residues,

Alkyl radicals which may be mentioned are substituted or unsubstituted, saturated or unsaturated C₂-C₂₄-alkylcarbonyl- chains such as ethylcarbonyl-, n-propylcarbonyl-, n-butylcarbonyl-, n-pentylcarbonyl-, n-hexylcarbonyl-, n-heptylcarbonyl-, n-octylcarbonyl-, n-nonylcarbonyl-, n-decylcarbonyl-, n-undecylcarbonyl-, n-dodecylcarbonyl-, n-tridecylcarbonyl-, n-tetradecylcarbonyl-, n-pentadecylcarbonyl-, n-hexadecylcarbonyl-, n-heptadecylcarbonyl-, n-octadecylcarbonyl-, n-nonadecylcarbonyl-, n-eicosylcarbonyl-, n-docosanylcarbonyl- or n-tetracosanylcarbonyl-, that contain one or more double bonds. Saturated or unsaturated C₁₀-C₂₂-Alkylcarbonylresidues such as n-decylcarbonyl-, n-undecylcarbonyl-, n-dodecylcarbonyl-, n-tridecylcarbonyl-, n-tetradecylcarbonyl-, n-pentadecylcarbonyl-, n-hexadecylcarbonyl-, n-heptadecylcarbonyl-, n-octadecylcarbonyl-, n-nonadecylcarbonyl-, n-eicosylcarbonyl-, n-docosanylcarbonyl- or n-tetracosanylcarbonyl- are preferred, which contain one or more double bonds. In particular privileged are saturated or unsaturated C₁₀-C₂₂-alkylcarbonyl-residue as C₁₀-alkylcarbonyl-, C₁₁-alkylcarbonyl-, C₁₂-alkylcarbonyl-, C₁₃-alkylcarbonyl-, C₁₄-alkylcarbonyl-, C₁₅-alkylcarbonyl-, C₁₆-alkylcarbonyl-, C₁₈-alkylcarbonyl-, C₂₀-alkylcarbonyl-, C₂₂-alkylcarbonyl- or C₂₄-alkylcarbonyl-residue, that contain one or more double bonds. In particular privileged are saturated or unsaturated C₁₆-C₂₂-alkyl-

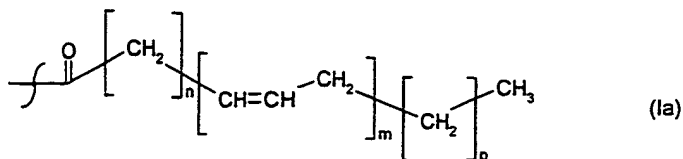
carbonylresidue as C₁₆-alkylcarbonyl-, C₁₈-alkylcarbonyl-, C₂₀-alkylcarbonyl- or C₂₂-alkylcarbonyl-residue, that contain one or more double bonds. The residues contain in particular two, three, four or five double bonds. Particularly preferred are residues of 20 or 22 carbon atoms having up to five double bonds, preferably three, four or five double bonds. All residues are derived from the mentioned corresponding fatty acids.

R³ indicates in structures of the general formula II hydrogen, saturated or unsaturated C₂-C₂₄-alkylcarbonyl.

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Substituted or unsubstituted, saturated or unsaturated C₂-C₂₄-alkylcarbonyl- residues are e. g. ethylcarbonyl-, n-propylcarbonyl-, n-butylcarbonyl-, n-pentylcarbonyl-, n-hexylcarbonyl-, n-heptylcarbonyl-, n-octylcarbonyl-, n-nonylcarbonyl-, n-decylcarbonyl-, n-undecylcarbonyl-, n-dodecylcarbonyl-, n-tridecylcarbonyl-, n-tetradecylcarbonyl-, n-pentadecylcarbonyl-, n-hexadecylcarbonyl-, n-heptadecylcarbonyl-, n-octadecylcarbonyl-, n-nonadecylcarbonyl-, n-eicosylcarbonyl-, n-docosanylcarbonyl- or n-tetracosanylcarbonyl-, having one or more double bonds. Preferred are saturated or unsaturated C₁₀-C₂₄-alkylcarbonyl residues as n-decylcarbonyl-, n-undecylcarbonyl-, n-dodecylcarbonyl-, n-tridecylcarbonyl-, n-tetradecylcarbonyl-, n-pentadecylcarbonyl-, n-hexadecylcarbonyl-, n-heptadecylcarbonyl-, n-octadecylcarbonyl-, n-nonadecylcarbonyl-, n-eicosylcarbonyl-, n-docosanylcarbonyl- or n-tetracosanylcarbonyl-, with one or more double bonds. In particular saturated or unsaturated C₁₀-C₂₄-alkylcarbonyl residues as C₁₀-alkylcarbonyl-, C₁₁-alkylcarbonyl-, C₁₂-alkylcarbonyl-, C₁₃-alkylcarbonyl-, C₁₄-alkylcarbonyl-, C₁₅-alkylcarbonyl-, C₁₆-alkylcarbonyl-, C₂₀-alkylcarbonyl-, C₂₂-alkylcarbonyl- or C₂₄-alkylcarbonyl-residues with one or more double bonds. In particular preferred are saturated or unsaturated C₁₆-C₂₂-alkylcarbonylresidue as C₁₆-alkylcarbonyl-, C₁₈-alkylcarbonyl-, C₂₀-alkylcarbonyl- or C₂₂-alkylcarbonyl-residues, with multiple double bonds. C₁₈-alkylcarbonyl-residues are particularly preferred, which contain one, two, three or four double bonds and C₂₀-alkylcarbonyl-residues, with three, four or five double bonds. All residues are derived from the corresponding fatty acids.

R^2 and R^3 indicates in structures of the general formula II independent of each other a residue of the general formula Ia



whereas the variables in the formula I and Ia are defined as: $n = 3, 4$ or 6 , $m = 3, 4$ or 5 and $p = 0$ or 3 . In particular: $n = 3$, $m = 4$ or 5 and $p = 0$ or 3 .

The abovementioned residues R^1 , R^2 and R^3 can be substituted with hydroxyl- or epoxy-groups or might contain also triple bonds.

- 10 According to the invention the used nucleic acid sequences are isolated nucleic sequences coding for polypeptides having C_{20} - $\Delta 5$ - or $\Delta 8$ desaturase or C_{18} - $\Delta 9$ -elongase activity.

- The according to inventive process synthesized substances of formula I which contain as residue R^1 the residue of formula II contain preferentially a mixture of different residues R^2 or R^3 . The residues are derived from different fatty acid molecules as short chain fatty acids with 4 to 6 C-atoms, mid-chain fatty acids having 8 to 12 C-atoms and long-chain fatty acids with 14 to 24 C-atoms, whereas the long-chain fatty acids are preferred. Said long chain fatty acids are derived preferentially from C_{18} - or C_{20} -poly unsaturated fatty acids having advantageously between two and five double bonds. In addition the backbone of formula I is also derived from such a aforementioned fatty acid which advantageously is also different from R^2 and R^3 . That means compounds which are produced by the inventive process are in one aspect of the invention triglycerides of different substituted or unsubstituted, saturated or unsaturated fatty acid ester or thioesters.

According to another aspect of the invention poly-unsaturated fatty acid esters (of the formula I) with 18, 20 or 22 fatty acid carbon atoms chain length with at least two double bonds, preferably three, four or five are particularly preferred.

In particular fatty acid molecules with three, four or five double bonds are preferred for the synthesis of eicosadienoic, eicosatrienoic, eicosatetraenoic (arachidonic-acid) and eicosapentanoic acid (C20:2n-6, Δ 11, 14; C20:3n-6, Δ 8, 11, 14; C20:4n-6, Δ 5, 8, 11, 14, C20:3n-3, Δ 11, 14, 17; C20:4n-3, Δ 8, 11, 14, 17; C20:5n-3, Δ 5, 8, 11, 14, 17) in the inventive process, whereas arachidonic acid and eicosapentaenoic acid are most preferred. We have found that this object is advantageously achieved by the combined expression of three isolated nucleic acid sequences according to the invention which encode for polypeptides having the following activities: a polypeptides with C20- Δ -8-desaturase activity, a C18- Δ -9-elongase activity, and a C20- Δ -5 desaturase activity.

This objective was achieved in particular by the co-expression of the isolated nucleic acid sequences according to the invention. C18 fatty acids with a double bond in Δ -9-position are elongated by the Δ -9-elongase advantageously used in the inventive process. By the Δ -8-desaturase used in the process a double in Δ -8-position is introduced into C20 fatty acids. In addition a double bond can be introduced into the fatty acid molecules in Δ -5-position by the Δ -5-desaturase.

The fatty acid ester of C₁₈-, C₂₀- and/or C₂₂-poly unsaturated fatty acids synthesized in the inventive process advantageously in form of their triglycerides as ester or thio-esters can be isolated from the producing organism for example from a microorganism or a plant in the form of an oil, lipid or lipid mixture for example as sphingolipids, phosphoglycerides, lipids, glycolipids such as glycosphingolipids, phospholipids such as phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine, phosphatidylglycerol, phosphatidylinositol or diphosphatidylglycerol, or as monoacylglyceride, diacylglyceride or triacylglyceride or as other fatty acid esters such as acetyl-Coenzym

A thioester, which contain saturated or unsaturated fatty acids preferably poly unsaturated fatty acids with at least two preferably at least three double bonds in the fatty acid molecule. In addition to the in form of the aforementioned esters bound fatty acids also fatty acids bound in other compounds can be produced or also free fatty acids can be produced by the inventive process.

In general the transgenic organisms for example transgenic microorganisms or plants used in the inventive process contain fatty acid esters or fatty acids in a distribution of nearly 80 to 90 % by weight of triacyl glycerides, 2 to 5 % by weight diacyl glycerides, 5 to 10 % by weight monoacyl glycerides, 1 to 5 % by weight free fatty acids and 2 to

8 % by weight phospholipids, whereas the total amount of the aforementioned compounds are all together a 100 % by weight.

In the inventive process(es) [the singular shall include the plural and vice versa] at
5 least 1 % by weight, preferably at least 2, 3, 4 or 5 % by weight, more preferably
at least 6, 7, 8, or 9 % by weight, most preferably 10, 20 or 30 % by weight of the
compounds of formula I referred to the total lipid content of the organism used in the
process are produced. Preferred starting material for the inventive process are linoleic
10 acid (C18:2) and/or linolenic acid (C18:3) which are transformed to the preferred end
products ARA or EPA. As for the inventive process organisms are used the product of
the process is not a product of one pure substance per se. It is a mixture of different
substances of formula I where one or more compounds are the major product and
others are only contained as side products. In the event that in an organism used in
15 the process linoleic and linolenic acid are available the end product is a mixture of ARA
and EPA. Advantageously the side products shall not exceed 20 % by weight referred
to the total lipid content of the organism, preferably the side products shall not exceed
15 % by weight, more preferably they shall not exceed 10 % by weight, most preferably
they shall not exceed 5 % by weight. Preferably organisms are used in the process
20 which contain as starting material either linoleic or linolenic acid so that as end product
of the process only ARA or EPA are produced. In the event EPA and ARA are
produced together, they should be produced in a ratio of at least 1:2 (EPA:ARA),
preferably of at least 1:3, more preferably of at least 1:4, most preferably of at least
1:5. In the event that a mixture of different fatty acids such as ARA and EPA are the
25 product of the inventive process said fatty acids can be further purified by method
known by a person skilled in the art such as distillation, extraction, crystallization at
low temperatures, chromatography or a combination of said methods.

Advantageously the invented method comprise the following steps:

- 30 a) expression of at least one nucleic acid sequence in a plant that codes for
an enzyme having Δ -9 elongase activity, and
- b) expression of at least one nucleic acid sequences which codes for a
C20-specific Δ -8 desaturase, and

- c) possibly the expression of a third nucleic acid sequence which codes for a C20-specific Δ -5 desaturase
- d) followed by the cultivation of the transgenic plants and seed harvest.

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In principle all host organisms can be used in the inventive process for example transgenic organisms such as plants like mosses; green, red, brown or blue algae; monocotyledons or dicotyledones. Advantageously oil producing transgenic organisms such as fungi, bacteria, algae, mosses or plants are used in the inventive processes described herein (for the invention the singular shall include the plural and vice versa), Additional advantageously organisms are animals or preferably plants or parts thereof. Fungi, yeasts or plants are preferably used, particularly preferably fungi or plants, very particularly preferably plants such as oilseed plants containing high amounts of lipid compounds such as rapeseed, poppy, mustard, hemp, castor bean, sesame, olive, calendula, punica, hazel nut, almond, macadamia, avocado, pumpkin, walnut, laurel, pistachio, primrose, canola, peanut, linseed, soybean, safflower, sunflower, borage or plants such as maize, wheat, rye, oat, triticale, rice, barley, cotton, manihot, pepper, tagetes, solanaceous plants such as potato, tobacco, eggplant, and tomato, Vicia species, pea, alfalfa, bushy plants (coffee, cacao, tea), Salix species, trees (oil palm, coconut) and perennial grasses and forage crops. Particularly preferred plants of the invention are oilseed plants rapeseed, poppy, mustard, hemp, castor bean, sesame, olive, calendula, punica, hazel nut, almond, macadamia, avocado, pumpkin, laurel, pistachio, primrose, canola, peanut, linseed, soybean, safflower, sunflower, borage or trees (oil palm, coconut). Most preferred are C_{18:2}- and/or C_{18:3}-fatty acid rich plants such as hemp, sesame, linseed, poppy, pumpkin, walnut, tobacco, cotton, safflower or sunflower.

Depending on the nucleic acid and/or the organism used in the inventive processes different compounds of the general formula I can be synthesized. In addition depending on the plant or fungi used in the process different mixtures of formula I compounds or single compounds such as arachidonic acid or eicosapentaenoic acid in free or bound form can be produced. In the event that in the inventive processes organism are used which have as precursor of the fatty acid synthesis preferably C_{18:2}- or C_{18:3}-fatty acids different poly unsaturated fatty acids can be synthesized for example starting

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from C_{18:2}-fatty acids γ -linoleic acid, dihomo- γ -linoleic acid or arachidonic acid can be produced or starting from C_{18:3}-fatty acids stearidonic acid, eicosatetraenoic acid or eicosapentaenoic acid can be produced. By influencing the activity of the different genes or their gene products different single compounds or compound mixtures can be produced. As living organisms are used in the inventive process the crude material that means crude lipids and/or oils isolated from the organisms preferably contain at least some starting compounds such as C_{18:2}- or C_{18:3}-fatty acids or their combination in the product and depending on the activity of the nucleic acid sequences and their gene products fatty acid intermediates of the biosynthesis chain. Said starting compounds or intermediates are in the product in a concentration of less than 20 or 15 % by weight, preferably less than 10, 9, 8, 7 or 6 % by weight, more preferably less than 5, 4, 3, 2 or 1 % by weight of the total fatty acids isolated from the used organism.

Transgenic plants are to be understood as meaning single plant cells and their cultures on solid media or in liquid culture, parts of plants and entire plants such as plant cell cultures, protoplasts from plants, callus cultures or plant tissues such as leaves, shoots, seeds, flowers, roots etc. Said transgenic plants can be cultivated for example on solid or liquid culture medium, in soil or in hydroponics.

After cultivation transgenic organisms preferably transgenic plants which are used in the inventive process can be brought to the market without isolating compounds of the general formula I. Preferably the compounds of the general formula I are isolated from the organisms in the form of their free fatty acids, their lipids or oils. The purification can be done by conventional methods such as squeezing and extraction of the plants or other methods instead of the extraction such as distillation, crystallization at low temperatures, chromatography or a combination of said methods. Advantageously the plants are grinded, heated and/or vaporized before the squeezing and extraction procedure. As solvent for the extraction solvents such as hexane are used. The isolated oils are further purified by acidification with for example phosphoric acid. The free fatty acids are produced from said oils or lipids by hydrolysis. Charcoal or diatom earth are used to remove dyes from the fluid. In another preferred embodiment of the inventive process the alkyl ester of the fatty acids are produced from the oils and lipids by transesterification with an enzyme or with conventional chemistry. A preferred method is the production of the alkyl ester in the presence of alcoholates of the

corresponding lower alcohols (C1 to C10 alcohols such as methanol, ethanol, propanol, butanol, hexanol etc.) such as methanolate or ethanolate. Therefore as the skilled worker knows the alcohol in the presence of a catalytic amount of a base such as NaOH or KOH is added to the oils or lipids.

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In a preferred form of the inventive process the lipids can be obtained in the usual manner after the organisms have been grown. To this end, the organisms can first be harvested and then disrupted, or they can be used directly. It is advantageous to extract the lipids with suitable solvents such as apolar solvents, for example hexane, 10 or polar solvents, for example ethanol, isopropanol, or mixtures such as hexane/isopropanol, phenol/chloroform/isoamyl alcohol, at temperatures between 0°C and 80°C, preferably between 20°C and 50°C. As a rule, the biomass is extracted with an excess of solvent, for example with an excess of solvent to biomass of 1:4. The solvent is subsequently removed, for example by distillation. The extraction may also be carried 15 out with supercritical CO₂. After the extraction, the remainder of the biomass can be removed, for example, by filtration. Standard methods for the extraction of fatty acids from plants and microorganisms are described in Bligh et al. (Can. J. Biochem. Physiol. 37, 1959: 911-917) or Vick et al. (Plant Physiol. 69, 1982: 1103-1108).

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The crude oil thus obtained can then be purified further, for example by removing cloudiness by adding polar solvents such as acetone or apolar solvents such as chloroform, followed by filtration or centrifugation. Further purification via columns or other techniques is also possible.

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To obtain the free fatty acids from the triglycerides, the latter are hydrolyzed in the customary manner, for example using NaOH or KOH.

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In the inventive process oils, lipids and/or free fatty acids or fractions thereof are produced. Said products can be used for the production of feed and food products, cosmetics or pharmaceuticals.

In principle all nucleic acids encoding polypeptides with Δ -8-desaturase, Δ -9-elongase and/or Δ -5-desaturase activity can be used in the inventive process. Preferably the nucleic acid sequences can be isolated for example from microorganism or plants such

as fungi like *Mortierella*, algae like *Euglena*, *Cryptocodinium* or *Isochrysis*, diatoms like *Phaeodactylum* or mosses like *Physcomitrella* or *Ceratodon*, but also non-human animals such as *Caenorhabditis* are possible as source for the nucleic acid sequences. Advantageous nucleic acid sequences according to the invention which encode

- 5 polypeptides having a Δ -8-desaturase, Δ -9-elongase and/or Δ -5-desaturase activity are originate from microorganisms or plants, advantageously *Phaeodactylum tricornutum*, *Ceratodon purpureus*, *Physcomitrella patens*, *Euglena gracilis* or *Isochrysis galbana*. *Euglena gracilis* or *Isochrysis galbana* are specific for the conversion of ω -3- or ω -6 fatty acids. Thus, the co expression of a Δ -9 elongase and a C20-specific
- 10 Δ -8-desaturase leads to the formation of eicosatrienoic acid (C20:6n-3, Δ 8, 11, 14) and eicosatetraenoic acid (C20:3n-4, Δ 8, 11, 14, 17). Co-expression of a third gene coding for a C20- Δ 5 specific desaturase leads to the production of Arachidonic acid (C20:6n-4, Δ 5, 8, 11, 14) or Eicosapentaenoic acid (C20:3n-5, Δ 5, 8, 11, 14, 17).

- 15 By derivative(s) of the sequences according to the invention is meant, for example, functional homologues of the polypeptides or enzymes encoded by SEQ ID NO: 2 or SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8 or SEQ ID NO: 10 which exhibit the same said specific enzymatic activity. This specific enzymatic activity allows advantageously the synthesis of unsaturated fatty acids having more than three double
- 20 bonds in the fatty acid molecule. By unsaturated fatty acids is meant in what follows diunsaturated or polyunsaturated fatty acids which possess double bonds. The double bonds may be conjugated or non conjugated. The said sequences encode enzymes which exhibit Δ -9 elongase, Δ -8-desaturase or Δ -5-desaturase activity.

- 25 The enzyme according to the invention, Δ -9 elongase, Δ -8-desaturase or Δ -5-desaturase, advantageously either elongates fatty acid chains with 18 carbon atoms (see SEQ ID NO: 2) or introduces a double bond into fatty acid residues of glycerolipids, free fatty acids or acyl-CoA fatty acids at position C₈-C₉ (see SEQ ID NO: 4) or at position C₅-C₆ (see SEQ ID NO: 6, SEQ ID NO: 8 or SEQ ID NO: 10).

- 30 The nucleic acid sequence(s) according to the invention (for purposes of the application the singular encompasses the plural and vice versa) or fragments thereof may advantageously be used for isolating other genomic sequences via homology screening.

The said derivatives may be isolated, for example, from other organisms, eukaryotic organisms such as plants, especially mosses, algae, dinoflagellates or fungi, preferably algae and mosses.

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Allele variants include in particular functional variants obtainable by deletion, insertion or substitution of nucleotides in the sequences depicted in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7 or SEQ ID NO: 9 the enzymatic activity of the derived synthesized proteins being retained.

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Starting from the DNA sequence described in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7 or SEQ ID NO: 9 or parts of said sequences such DNA sequences can be isolated using, for example, normal hybridization methods or the PCR technique from other eukaryotes such as those identified above for example.

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These DNA sequences hybridize under standard conditions with the said sequences. For hybridization use is advantageously made of short oligonucleotides of the conserved regions of an average length of about 15 to 70 bp, preferably of about 17 to 60 bp, more preferably of about 19 to 50 bp, most preferably of about 20 to 40 bp, for example, which can be determined by comparisons with other desaturase or elongase

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genes in the manner known to those skilled in the art. The histidine box sequences are advantageously employed. However, longer fragments of the nucleic acids according to the invention or the complete sequences may also be used for hybridization.

Depending on the nucleic acid employed: oligonucleotide, longer fragment or complete sequence, or depending on which type of nucleic acid, DNA or RNA, is used for

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hybridization these standard conditions vary. Thus, for example, the melting temperatures of DNA:DNA hybrids are approximately 10 °C lower than those of DNA:RNA hybrids of the same length.

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By standard conditions is meant, for example, depending on the nucleic acid in question temperatures between 42 °C and 58 °C in an aqueous buffer solution having a concentration of between 0.1 and 5 x SSC (1 X SSC = 0.15 M NaCl, 15 mM sodium citrate, pH 7.2) or additionally in the presence of 50 % formamide, such as by way of example 42 °C in 5 x SSC, 50 % formamide. Hybridization conditions for DNA:DNA hybrids are advantageously 0.1 x SSC and temperatures between approximately 20 °C

and 45 °C, preferably between approximately 30 °C and 45 °C. For DNA:RNA hybrids the hybridization conditions are advantageously 0.1 x SSC and temperatures between approximately 30 °C and 55 °C, preferably between approximately 45 °C and 55 °C. These specified temperatures for hybridization are melting temperature values
5 calculated by way of example for a nucleic acid having a length of approximately 100 nucleotides and a G + C content of 50 % in the absence of formamide. The experimental conditions for DNA hybridization are described in relevant genetics textbooks such as by way of example Sambrook et al., „Molecular Cloning“, Cold Spring Harbor Laboratory, 1989, and may be calculated by formulae known to those
10 skilled in the art, for example as a function of the length of the nucleic acids, the nature of the hybrids or the G + C content. Those skilled in the art may draw on the following textbooks for further information on hybridization: Ausubel et al. (eds), 1985, Current Protocols in Molecular Biology, John Wiley & Sons, New York; Hames and Higgins (eds), 1985, Nucleic Acids Hybridization: A Practical Approach, IRL Press at Oxford
15 University Press, Oxford; Brown (ed), 1991, Essential Molecular Biology: A Practical Approach, IRL Press at Oxford University Press, Oxford.

Furthermore, by derivatives is meant homologues of the sequences SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7 and SEQ ID NO: 9, for example
20 eukaryotic homologues, truncated sequences, single-stranded DNA of the encoding and nonencoding DNA sequence or RNA of the encoding and nonencoding DNA sequence.

In addition, by homologues of the sequences SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7 and SEQ ID NO: 9 is meant derivatives such as by way of
25 example promoter variants. These variants may be modified by one or more nucleotide exchanges, by insertion(s) and/or deletion(s) without, however, adversely affecting the functionality or efficiency of the promoters. Furthermore, the promoters can have their efficiency increased by altering their sequence or be completely replaced by more
30 effective promoters even of foreign organisms.

By derivatives is also advantageously meant variants whose nucleotide sequence has been altered in the region from -1 to -2000 ahead of the start codon in such a way that the gene expression and/or the protein expression is modified, preferably

increased. Furthermore, by derivatives is also meant variants which have been modified at the 3' end.

5 The nucleic acid sequences according to the invention which encode a Δ -8-desaturase, a Δ -5-desaturase and/or a Δ -9-elongase may be produced by synthesis or obtained naturally or contain a mixture of synthetic and natural DNA components as well as consist of various heterologous Δ -8-desaturase, Δ -5-desaturase and/or Δ -9-elongase gene segments from different organisms. In general, synthetic nucleotide sequences are produced with codons which are preferred by the corresponding host
10 organisms, plants for example. This usually results in optimum expression of the heterologous gene. These codons preferred by plants may be determined from codons having the highest protein frequency which are expressed in most of the plant species of interest. An example concerning *Corynebacterium glutamicum* is provided in Wada et al. (1992) *Nucleic Acids Res.* 20:2111-2118). Such experiments can be carried out
15 using standard methods and are known to the person skilled in the art.

Functionally equivalent sequences which encode the Δ -8-desaturase, Δ -5-desaturase and/or Δ -9-elongase gene are those derivatives of the sequence according to the invention which despite differing nucleotide sequence still possess the desired
20 functions, that is to say the enzymatic activity and specific selectivity of the proteins. Thus, functional equivalents include naturally occurring variants of the sequences described herein as well as artificial ones, e.g. artificial nucleotide sequences adapted to the codon use of a plant which have been obtained by chemical synthesis.

25 In addition, artificial DNA sequences are suitable, provided, as described above, they mediate the desired property, for example an increase in the content of Δ -8 and/or Δ -5 double bonds in fatty acids, oils or lipids in organisms such as in a plant by over-expression of the Δ -8-and/or Δ -5-desaturase gene in preferably in crop plants. Such artificial DNA sequences can exhibit Δ -8 and/or Δ -5-desaturase and/or Δ -9-elongase
30 activity, for example by back-translation of proteins constructed by means of molecular modeling, or be determined by in vitro selection. Possible techniques for in vitro evolution of DNA to modify or improve the DNA sequences are described in Patten, P.A. et al., *Current Opinion in Biotechnology* 8, 724-733 (1997) or in Moore, J.C. et al., *Journal of Molecular Biology* 272, 336-347 (1997). Particularly suitable are encoding

DNA sequences which are obtained by back-translation of a polypeptide sequence in accordance with the codon use specific to the host plant. Those skilled in the art familiar with the methods of plant genetics can easily determine the specific codon use by computer analyses of other known genes of the plant to be transformed.

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Other suitable equivalent nucleic acid sequences which may be mentioned are sequences that encode fusion proteins, a component of the fusion protein being a Δ -8- and/or a Δ -5-desaturase polypeptide and/or a Δ -9 elongase polypeptide or a functionally equivalent part thereof. The second part of the fusion protein can be, for example, another polypeptide having enzymatic activity or an antigenic polypeptide sequence by means of which it is possible to demonstrate Δ -8- and/or Δ -5-desaturase or Δ -9-elongase expression (e.g. myc tag or his tag). Preferably, however, this is a regulatory protein sequence, such as by way of example a signal sequence for the endoplasmic reticulum (= ER) which directs the Δ -8- and/or Δ -5-desaturase protein and/or the Δ -9-elongase protein to the desired point of action, or regulatory sequences which influence the expression of the nucleic acid sequence according to the invention, such as promoters or terminators. In another preferred embodiment the second part of the fusion protein is a plastidial targeting sequence as described by Napier J.A. [Targeting of foreign proteins to the chloroplast, *Methods Mol. Biol.*, 49, 1995: 369 - 376]. A preferred used vector comprising said plastidial targeting sequence is disclosed by Colin Lazarus [Guerineau F., Woolston S., Brooks L., Mullineaux P. "An expression cassette for targeting foreign proteins into chloroplast; *Nucleic. Acids Res.*, Dec 9, 16 (23), 1988: 11380].

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Advantageously, the Δ -8-desaturase and Δ -9-elongase and/or the Δ -5-desaturase genes in the method according to the invention may be combined with other genes for fatty acid biosynthesis. Examples of such genes are the acyl transferases, other desaturases or elongases such as Δ -4-, Δ -5- or Δ -6-desaturases or ω -3- and/or ω -6-specific desaturases such as Δ -12 (for C_{18} fatty acids), Δ -15 (for C_{18} fatty acids) or Δ -19 (for C_{22} fatty acids) and/or such as Δ -5- or Δ -6-elongases. For in vivo and especially in vitro synthesis combination with e.g. NADH cytochrome B5 reductases which can take up or release reduction equivalents is advantageous.

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By the amino acid sequences according to the invention is meant proteins which contain an amino acid sequence depicted in the sequences SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8 and SEQ ID NO: 10 or a sequence obtainable therefrom by substitution, inversion, insertion or deletion of one or more amino acid groups (such sequences are derivatives of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8 and/or SEQ ID NO: 10), whereas the enzymatic activities of the proteins depicted in SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8 and SEQ ID NO: 10 being retained or not substantially reduced, that is they still possess the same enzymatic specificity. By „not substantially reduced“ or „the same enzymatic activity“ is meant all enzymes which still exhibit at least 10 %, preferably 20 %, particularly preferably 30 %, of the enzymatic activity of the initial enzyme obtained from the wild type source organism such as organisms of the genus *Physcomitrella*, *Ceratodon*, *Borago*, *Thraustochytrium*, *Schizochytrium*, *Phytophthora*, *Mortierella*, *Caenorhabditis*, *Aleuritia*, *Muscarioides*, *Isochrysis*, *Phaeodactylum*, *Cryptothecodinium* or *Eugenia* preferred source organisms are organisms such as the species *Eugenia gracilis*, *Isochrysis galbana*, *Phaeodactylum tricomutum*, *Caenorhabditis elegans*, *Thraustochytrium*, *Phytophthora infestans*, *Ceratodon purpureus*, *Isochrysis galbana*, *Aleuritia farinosa*, *Muscarioides vialii*, *Mortierella alpina*, *Borago officinalis* or *Physcomitrella patens*. For the estimation of an enzymatic activity which is “not substantially reduced” or which has the “same enzymatic activity” the enzymatic activity of the derived sequences are determined and compared with the wild type enzyme activities. In doing this, for example, certain amino acids may be replaced by others having similar physicochemical properties (space filling, basicity, hydrophobicity, etc.). For example, arginine residues are exchanged for lysine residues, valine residues for isoleucine residues or aspartic acid residues for glutamic acid residues. However, one or more amino acids may also be swapped in sequence, added or removed, or a plurality of these measures may be combined with one another.

By derivatives is also meant functional equivalents which in particular also contain natural or artificial mutations of an originally isolated sequence encoding Δ -8-desaturase, a Δ -9-elongase and/or a Δ -5-desaturase which continue to exhibit the desired function, that is the enzymatic activity and substrate selectivity thereof is not substantially reduced. Mutations comprise substitutions, additions, deletions, exchanges or insertions of one or more nucleotide residues. Thus, for example, the present invention

also encompasses those nucleotide sequences which are obtained by modification of the Δ -8-desaturase nucleotide sequence, the Δ -5-desaturase nucleotide sequence and/or the Δ -9-elongase nucleotide sequence used in the inventive processes. The aim of such a modification may be, e.g., to further bound the encoding sequence
5 contained therein or also, e.g., to insert further restriction enzyme interfaces.

Functional equivalents also include those variants whose function by comparison as described above with the initial gene or gene fragment is weakened (= not substantially reduced) or reinforced (= enzyme activity higher than the activity of the initial enzyme,
10 that is activity is higher than 100 %, preferably higher than 110 %, particularly preferably higher than 130 %).

At the same time the nucleic acid sequence may, for example, advantageously be a DNA or cDNA sequence. Suitable encoding sequences for insertion into an expression
15 cassette according to the invention include by way of example those which encode a Δ -8-desaturase, a Δ -5-desaturase and/or a Δ -9-elongase with the sequences described above and lend the host the ability to overproduce fatty acids, oils or lipids having double bonds in the Δ -8-position and Δ -5-position, it being advantageous when at the same time fatty acids having at least four double bonds are produced. These
20 sequences may be of homologous or heterologous origin.

By the expression cassette (= nucleic acid construct or fragment or gene construct) according to the invention is meant the sequences specified in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7 and/or SEQ ID NO: 9 which result from the
25 genetic code and/or derivatives thereof which are functionally linked with one or more regulation signals advantageously to increase the gene expression and which control the expression of the encoding sequence in the host cell. These regulatory sequences should allow the selective expression of the genes and the protein expression. Depending on the host organism this may mean, for example, that the gene is
30 expressed and/or overexpressed only after induction or that it is expressed and/or overexpressed immediately. Examples of these regulatory sequences are sequences to which inducers or repressors bind and in this way regulate the expression of the nucleic acid. In addition to these new regulation sequences or instead of these sequences the natural regulation of these sequences ahead of the actual structural

genes may still be present and optionally have been genetically modified so that natural regulation was switched off and the expression of the genes increased. However, the gene construct can also be built up more simply, that is no additional regulation signals have been inserted ahead of the nucleic acid sequence or derivatives thereof and the natural promoter with its regulation has not been removed. Instead of this the natural regulation sequence was mutated in such a way that no further regulation ensues and/or the gene expression is heightened. These modified promoters in the form of part sequences (= promoter containing parts of the nucleic acid sequences according to the invention) can also be brought on their own ahead of the natural gene to increase the activity. In addition, the gene construct may advantageously also contain one or more so-called enhancer sequences functionally linked to the promoter which allow enhanced expression of the nucleic acid sequence. At the 3' end of the DNA sequences additional advantageous sequences may also be inserted, such as further regulatory elements or terminators. The Δ -8- and/or Δ -5-desaturase gene and/or the Δ -9-elongase gene may be present in one or more copies in the expression cassette (= gene construct).

As described above, the regulatory sequences or factors can preferably positively influence and so increase the gene expression of the introduced genes. Thus, reinforcement of the regulatory elements advantageously on the transcription level may be effected by using powerful transcription signals such as promoters and/or enhancers. However, in addition reinforcement of translation is also possible, for example by improving the stability of the mRNA.

Suitable promoters in the expression cassette are in principle all promoters which can control the expression of foreign genes in organisms such as microorganisms like protozoa such as ciliates, algae such as green, brown, red or blue algae such as Euglenia, bacteria such as gram-positive or gram-negative bacteria, yeasts such as Saccharomyces, Pichia or Schizosaccharomyces or fungi such as Mortierella, Thraustochytrium or Schizochytrium or plants such as Aleuritia, advantageously in plants or fungi. Use is preferably made in particular of plant promoters or promoters derived from a plant virus. Advantageous regulation sequences for the method according to the invention are found for example in promoters such as cos, tac, trp, tet, trp-tet, lpp, lac, lpp-lac, lacI^q, T7, T5, T3, gal, trc, ara, SP6, λ -P_R or in λ -P_L promo-

ters which are employed advantageously in gram-negative bacteria. Other advantageous regulation sequences are found, for example, in the gram-positive promoters amy and SPO2, in the yeast or fungal promoters ADC1, MF α , AC, P-60, CYC1, GAPDH, TEF, rp28, ADH or in the plant promoters CaMV/35S [Franck et al., Cell 21(1980) 285-294], SSU, OCS, lib4, STLS1, B33, nos (= Nopaline Synthase Promoter) or in the ubiquitin or phaseolin promoter. The expression cassette may also contain a chemically inducible promoter by means of which the expression of the exogenous $\Delta 8$ - and/or $\Delta 5$ -desaturase gene and/or the $\Delta 9$ -elongase gene in the organisms can be controlled advantageously in the plants at a particular time. Advantageous plant promoters of this type are by way of example the PRP1 promoter [Ward et al., Plant. Mol. Biol. 22(1993), 361-366], a promoter inducible by benzenesulfonamide (EP 388 186), a promoter inducible by tetracycline [Gatz et al., (1992) Plant J. 2, 397-404], a promoter inducible by salicylic acid (WO 95/19443), a promoter inducible by abscisic acid (EP 335 528) and a promoter inducible by ethanol or cyclohexanone (WO93/21334). Other examples of plant promoters which can advantageously be used are the promoter of cytosolic FBPase from potato, the ST-LSI promoter from potato (Stockhaus et al., EMBO J. 8 (1989) 2445-245), the promoter of phosphoribosyl pyrophosphate amidotransferase from Glycine max (see also gene bank accession number U87999) or a nodiene-specific promoter as described in EP 249 676. Particularly advantageous are those plant promoters which ensure expression in tissues or plant parts/organs in which fatty acid biosynthesis or the precursor stages thereof occurs, as in endosperm or in the developing embryo for example. Particularly noteworthy are advantageous promoters which ensure seed-specific expression such as by way of example the USP promoter or derivatives thereof, the LEB4 promoter, the phaseolin promoter or the napin promoter. The particularly advantageous USP promoter cited according to the invention or its derivatives mediate very early gene expression in seed development [Baeumlein et al., Mol Gen Genet, 1991, 225 (3): 459-67]. Other advantageous seed-specific promoters which may be used for monocotyledonous or dicotyledonous plants are the promoters suitable for dicotyledons such as napin gene promoters, likewise cited by way of example, from oilseed rape (US 5,608,152), the oleosin promoter from Arabidopsis (WO 98/45461), the phaseolin promoter from Phaseolus vulgaris (US 5,504,200), the Bce4 promoter from Brassica (WO 91/13980) or the leguminous B4 promoter (LeB4, Baeumlein et al., Plant J., 2, 2, 1992: 233 - 239) or promoters suitable for monocotyledons such as the promoters of the lpt2 or

lpt1 gene in barley (WO 95/15389 and WO 95/23230) or the promoters of the barley hordeine gene, the rice glutelin gene, the rice oryzin gene, the rice prolamin gene, the wheat gliadin gene, the white glutelin gene, the corn zein gene, the oats glutelin gene, the sorghum kasirin gene or the rye secalin gene which are described in WO99/16890.

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Furthermore, particularly preferred are those promoters which ensure the expression in tissues or plant parts in which, for example, the biosynthesis of fatty acids, oils and lipids or the precursor stages thereof takes place. Particularly noteworthy are promoters which ensure a seed-specific expression. Noteworthy are the promoter of the

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napin gene from oilseed rape (US 5,608,152), the USP promoter from *Vicia faba* (USP = unknown seed protein, Baeumlein et al., Mol Gen Genet, 1991, 225 (3): 459-67), the promoter of the oleosin gene from *Arabidopsis* (WO98/45461), the phaseolin promoter (US 5,504,200) or the promoter of the legumin B4 gene (LeB4; Baeumlein et al., 1992, Plant Journal, 2 (2): 233-9). Other promoters to be mentioned are that

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of the lpt2 or lpt1 gene from barley (WO95/15389 and WO95/23230) which mediate seed-specific expression in monocotyledonous plants. Other advantageous seed

specific promoters are promoters such as the promoters from rice, corn or wheat disclosed in WO 99/16890 or Amy32b, Amy6-6 or aleurain (US 5,677,474), Bce4 (rape, US 5,530,149), glycinin (soy bean, EP 571 741), phosphoenol pyruvate carboxylase (soy bean, JP 06/62870), ADR12-2 (soy bean, WO 98/08962), isocitrate lyase (rape, US 5,689,040) or β -amylase (barley, EP 781 849).

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As described above, the expression construct (= gene construct, nucleic acid construct) may contain yet other genes which are to be introduced into the organisms.

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These genes can be subject to separate regulation or be subject to the same regulation region as the Δ -8- and/or Δ -5-desaturase gene and/or the Δ -9-elongase gene.

These genes are by way of example other biosynthesis genes, advantageously for fatty acid biosynthesis, which allow increased synthesis. Examples which may be mentioned are the genes for Δ -15-, Δ -12-, Δ -9-, Δ -5-, Δ -4-desaturase, α -ketoacyl

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reductases, α -ketoacyl synthases, elongases or the various hydroxylases and acyl-ACP thioesterases. The desaturase genes are advantageously used in the nucleic acid construct.

In principle all natural promoters with their regulation sequences can be used like those named above for the expression cassette according to the invention and the method according to the invention. Over and above this, synthetic promoters may also advantageously be used.

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In the preparation of an expression cassette various DNA fragments can be manipulated in order to obtain a nucleotide sequence which usefully reads in the correct direction and is equipped with a correct reading raster. To connect the DNA fragments (= nucleic acids according to the invention) to one another adaptors or linkers may be attached to the fragments.

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The promoter and the terminator regions can usefully be provided in the transcription direction with a linker or polylinker containing one or more restriction points for the insertion of this sequence. Generally, the linker has 1 to 10, mostly 1 to 8, preferably 2 to 6, restriction points. In general the size of the linker inside the regulatory region is less than 100 bp, frequently less than 60 bp, but at least 5 bp. The promoter may be both native or homologous as well as foreign or heterologous to the host organism, for example to the host plant. In the 5'-3' transcription direction the expression cassette contains the promoter, a DNA sequence which encodes a Δ -8-desaturase gene, a Δ -5-desaturase gene and/or a Δ -9-elongase gene and a region for transcription termination. Different termination regions can be exchanged for one another in any desired fashion.

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Furthermore, manipulations which provide suitable restriction interfaces or which remove excess DNA or restriction interfaces can be employed. Where insertions, deletions or substitutions, such as transitions and transversions, come into consideration, *in vitro* mutagenesis, primer repair, restriction or ligation may be used. In suitable manipulations such as restriction, chewing back or filling of overhangs for blunt ends complementary ends of the fragments can be provided for the ligation.

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For an advantageous high expression the attachment of the specific ER retention signal SEKDEL inter alia can be of importance (Schouten, A. et al., Plant Mol. Biol. 30 (1996), 781-792). In this way the average expression level is tripled or even quadrupled. Other retention signals which occur naturally in plant and animal proteins located

in the ER may also be employed for the construction of the cassette. In another preferred embodiment a plastidial targeting sequence is used as described by Napier J.A. [Targeting of foreign proteins to the chloroplast, *Methods Mol. Biol.*, 49, 1995: 369-376]. A preferred used vector comprising said plastidial targeting sequence is disclosed by Colin Lazarus [Guerineau F., Woolston S., Brooks L., Mullineaux P. "An expression cassette for targeting foreign proteins into chloroplast; *Nucleic. Acids Res.*, Dec 9, 16 (23), 1988: 11380].

Preferred polyadenylation signals are plant polyadenylation signals, preferably those which substantially correspond to T-DNA polyadenylation signals from *Agrobacterium tumefaciens*, in particular gene 3 of the T-DNA (octopine synthase) of the Ti plasmid pTiACH5 (Gielen et al., *EMBO J.* 3 (1984), 835 et seq.) or corresponding functional equivalents.

An expression cassette is produced by fusion of a suitable promoter with a suitable Δ -8- and/or Δ -5-desaturase DNA sequence and/or a suitable Δ -9-elongase DNA sequence together with a polyadenylation signal by common recombination and cloning techniques as described, for example, in T. Maniatis, E.F. Fritsch and J. Sambrook, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989) as well as in T.J. Silhavy, M.L. Berman and L.W. Enquist, *Experiments with Gene Fusions*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984) and in Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Greene Publishing Assoc. and Wiley-Interscience (1987).

In the preparation of an expression cassette various DNA fragments can be manipulated to produce a nucleotide sequence which usefully reads in the correct direction and is equipped with a correct reading raster. Adapters or linkers can be attached to the fragments for joining the DNA fragments.

The promoter and the terminator regions can usefully be provided in the transcription direction with a linker or polylinker containing one or more restriction points for the insertion of this sequence. Generally, the linker has 1 to 10, mostly 1 to 8, preferably 2 to 6, restriction points. In general the size of the linker inside the regulatory region is less than 100 bp, frequently less than 60 bp, but at least 5 bp. The promoter may be

both native or homologous as well as foreign or heterologous to the host organism, for example to the host plant. In the 5'-3' transcription direction the expression cassette contains the promoter, a DNA sequence which either encodes a Δ -8- and/or Δ -5-desaturase gene and/or a Δ -9-elongase gene and a region for transcription termination.

5 Different termination regions can be exchanged for one another in any desired fashion.

In the preparation of an expression cassette various DNA fragments can be manipulated to produce a nucleotide sequence which usefully reads in the correct direction and is equipped with a correct reading raster. Adapters or linkers can be attached to the fragments for joining the DNA fragments.

The DNA sequences encoding the nucleic acid sequences used in the inventive processes such as the Δ -8-desaturase from *Eugenia gracilis*, the Δ -9-elongase from *Isochrysis galbana* and/or the Δ -5-desaturase for example from *Caenorhabditis* 15 *elegans*, *Mortierella alpina*, *Borage officinalis* or *Physcomitrella patens* contain all the sequence characteristics needed to achieve correct localization of the site of fatty acid, lipid or oil biosynthesis. Accordingly, no further targeting sequences are needed per se. However, such a localization may be desirable and advantageous and hence artificially modified or reinforced so that such fusion constructs are also a preferred 20 advantageous embodiment of the invention.

Particularly preferred are sequences which ensure targeting in plastids. Under certain circumstances targeting into other compartments (reported in: Kermode, Crit. Rev. Plant Sci. 15, 4 (1996), 285-423) may also be desirable, e.g. into vacuoles, the 25 mitochondrion, the endoplasmic reticulum (ER), peroxisomes, lipid structures or due to lack of corresponding operative sequences retention in the compartment of origin, the cytosol.

Advantageously, the nucleic acid sequences according to the invention or the gene 30 construct together with at least one reporter gene are cloned into an expression cassette which is introduced into the organism via a vector or directly into the genome. This reporter gene should allow easy detection via a growth, fluorescence, chemical, bioluminescence or resistance assay or via a photometric measurement. Examples of reporter genes which may be mentioned are antibiotic- or herbicide-resistance genes,

hydrolase genes, fluorescence protein genes, bioluminescence genes, sugar or nucleotide metabolic genes or biosynthesis genes such as the Ura3 gene, the *llv2* gene, the luciferase gene, the β -galactosidase gene, the *gfp* gene, the 2-des-oxyglucose-6-phosphate phosphatase gene, the β -glucuronidase gene, β -lactamase gene, the neomycin phosphotransferase gene, the hygromycin phosphotransferase gene or the BASTA (= gluphosinate-resistance) gene. These genes permit easy measurement and quantification of the transcription activity and hence of the expression of the genes. In this way genome positions may be identified which exhibit differing productivity.

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In a preferred embodiment an expression cassette comprises upstream, i.e. at the 5' end of the encoding sequence, a promoter and downstream, i.e. at the 3' end, a polyadenylation signal and optionally other regulatory elements which are operably linked to the intervening encoding sequence for Δ -8-desaturase, Δ -9-elongase and/or Δ -5-desaturase DNA sequence. By an operable linkage is meant the sequential arrangement of promoter, encoding sequence, terminator and optionally other regulatory elements in such a way that each of the regulatory elements can fulfill its function in the expression of the encoding sequence in due manner. The sequences preferred for operable linkage are targeting sequences for ensuring subcellular localization in plastids. However, targeting sequences for ensuring subcellular localization in the mitochondrion, in the endoplasmic reticulum (= ER), in the nucleus, in oil corpuscles or other compartments may also be employed as well as translation promoters such as the 5' lead sequence in tobacco mosaic virus (Gallie et al., Nucl. Acids Res. 15 (1987), 8693 -8711).

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An expression cassette may, for example, contain a constitutive promoter or a tissue-specific promoter (preferably the USP or napin promoter) the gene to be expressed and the ER retention signal. For the ER retention signal the KDEL amino acid sequence (lysine, aspartic acid, glutamic acid, leucine) or the KKX amino acid sequence (lysine-lysine-X-stop, wherein X means every other known amino acid) is preferably employed.

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For expression in a prokaryotic or eukaryotic host organism, for example a microorganism such as a fungus or a plant the expression cassette is advantageously inserted

into a vector such as by way of example a plasmid, a phage or other DNA which allows optimum expression of the genes in the host organism. Examples of suitable plasmids are: in *E. coli* pLG338, pACYC184, pBR series such as e.g. pBR322, pUC series such as pUC18 or pUC19, M113mp series, pKC30, pRep4, pHS1, pHS2, pPLc236, pMBL24, pLG200, pUR290, pIN-III¹¹³-B1, λ gt11 or pBdCl; in *Streptomyces* pIJ101, pIJ364, pIJ702 or pIJ361; in *Bacillus* pUB110, pC194 or pBD214; in *Corynebacterium* pSA77 or pAJ667; in fungi pALS1, pIL2 or pBB116; other advantageous fungal vectors are described by Romanos, M.A. et al., [(1992) „Foreign gene expression in yeast: a review“, *Yeast* 8: 423-488] and by van den Hondel, C.A.M.J.J. et al. [(1991) „Heterologous gene expression in filamentous fungi“ as well as in *More Gene Manipulations in Fungi* [J.W. Bennet & L.L. Lasure, eds., pp. 396-428: Academic Press: San Diego] and in „Gene transfer systems and vector development for filamentous fungi“ [van den Hondel, C.A.M.J.J. & Punt, P.J. (1991) in: *Applied Molecular Genetics of Fungi*, Peberdy, J.F. et al., eds., pp. 1-28, Cambridge University Press: Cambridge]. Examples of advantageous yeast promoters are 2 μ M, pAG-1, YE6, YE13 or pEMBLye23. Examples of algal or plant promoters are pLGV23, pGHlac⁺, pBIN19, pAK2004, pVKH or pDH51 (see Schmidt, R. and Willmitzer, L., 1988). The vectors identified above or derivatives of the vectors identified above are a small selection of the possible plasmids. Further plasmids are well known to those skilled in the art and may be found, for example, in the book *Cloning Vectors* (Eds. Pouwels P.H. et al. Elsevier, Amsterdam-New York-Oxford, 1985, ISBN 0 444 904018). Suitable plant vectors are described inter alia in „Methods in Plant Molecular Biology and Biotechnology“ (CRC Press), Ch. 6/7, pp. 71-119. Advantageous vectors are known as shuttle vectors or binary vectors which replicate in *E. coli* and *Agrobacterium*.

By vectors is meant with the exception of plasmids all other vectors known to those skilled in the art such as by way of example phages, viruses such as SV40, CMV, baculovirus, adenovirus, transposons, IS elements, phasmids, phagemids, cosmids, linear or circular DNA. These vectors can be replicated autonomously in the host organism or be chromosomally replicated, chromosomal replication being preferred.

In a further embodiment of the vector the expression cassette according to the invention may also advantageously be introduced into the organisms in the form of a linear DNA and be integrated into the genome of the host organism by way of

heterologous or homologous recombination. This linear DNA may be composed of a linearized plasmid or only of the expression cassette as vector or the nucleic acid sequences according to the invention.

- 5 In a further advantageous embodiment the nucleic acid sequence according to the invention can also be introduced into an organism on its own.

If in addition to the nucleic acid sequence according to the invention further genes are to be introduced into the organism, all together with a reporter gene in a single vector
10 or each single gene with a reporter gene in a vector in each case can be introduced into the organism, whereby the different vectors can be introduced simultaneously or successively.

The vector advantageously contains at least one copy of the nucleic acid sequences
15 according to the invention and/or the expression cassette (= gene construct) according to the invention.

By way of example the plant expression cassette can be installed in the pRT transformation vector ((a) Toepfer et al., 1993, *Methods Enzymol.*, 217: 66-78; (b) Toepfer
20 et al. 1987, *Nucl. Acids. Res.* 15: 5890 ff.).

Alternatively, a recombinant vector (= expression vector) can also be transcribed and translated in vitro, e.g. by using the T7 promoter and the T7 RNA polymerase.

- 25 Expression vectors employed in prokaryotes frequently make use of inducible systems with and without fusion proteins or fusion oligopeptides, wherein these fusions can ensue in both N-terminal and C-terminal manner or in other useful domains of a protein. Such fusion vectors usually have the following purposes: i.) to increase the RNA expression rate; ii.) to increase the achievable protein synthesis rate; iii.) to
30 increase the solubility of the protein; iv.) or to simplify purification by means of a binding sequence usable for affinity chromatography. Proteolytic cleavage points are also frequently introduced via fusion proteins which allows cleavage of a portion of the fusion protein and purification. Such recognition sequences for proteases are recognized, e.g. factor Xa, thrombin and enterokinase.

Typical advantageous fusion and expression vectors are pGEX [Pharmacia Biotech Inc; Smith, D.B. and Johnson, K.S. (1988) *Gene* 67: 31-40], pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ) which contains
5 glutathione S-transferase (GST), maltose binding protein or protein A.

Other examples of *E. coli* expression vectors are pTrc [Amann et al., (1988) *Gene* 69:301-315] and pET vectors [Studier et al., *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California (1990) 60-89; Stratagene,
10 Amsterdam, The Netherlands].

Other advantageous vectors for use in yeast are pYepSec1 (Baldari, et al., (1987) *Embo J.* 6:229-234), pMFa (Kurjan and Herskowitz, (1982) *Cell* 30:933-943), pJRY88 (Schultz et al., (1987) *Gene* 54:113-123), and pYES derivatives (Invitrogen Corporation, San Diego, CA). Vectors for use in filamentous fungi are described in: van den
15 Hondel, C.A.M.J.J. & Punt, P.J. (1991) „Gene transfer systems and vector development for filamentous fungi“, in: *Applied Molecular Genetics of Fungi*, J.F. Peberdy, et al., eds., pp. 1-28, Cambridge University Press: Cambridge.

20 Alternatively, insect cell expression vectors can also be advantageously utilized, e.g. for expression in Sf 9 cells. These are e.g. the vectors of the pAc series (Smith et al. (1983) *Mol. Cell Biol.* 3:2156-2165) and the pVL series (Lucklow and Summers (1989) *Virology* 170:31-39).

25 Furthermore, plant cells or algal cells can advantageously be used for gene expression. Examples of plant expression vectors may be found in Becker, D., et al. (1992) „New plant binary vectors with selectable markers located proximal to the left border“, *Plant Mol. Biol.* 20: 1195-1197 or in Bevan, M.W. (1984) „Binary *Agrobacterium* vectors for plant transformation“, *Nucl. Acid. Res.* 12: 8711-8721.

30 Furthermore, the nucleic acid sequences may also be expressed in mammalian cells, advantageously in nonhuman mammalian cells. Examples of corresponding expression vectors are pCDM8 and pMT2PC referred to in: Seed, B. (1987) *Nature* 329:840 or Kaufman et al. (1987) *EMBO J.* 6: 187-195). At the same time promoters preferred

for use are of viral origin, such as by way of example promoters of polyoma, adenovirus 2, cytomegalovirus or simian virus 40. Other prokaryotic and eukaryotic expression systems are referred to in chapters 16 and 17 of Sambrook et al., *Molecular Cloning: A Laboratory Manual*. 2nd, ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

The host organism (= transgenic organism) advantageously contains at least one copy of the nucleic acid according to the invention and/or of the nucleic acid construct according to the invention.

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The introduction of the nucleic acids according to the invention, the expression cassette or the vector into organisms, plants for example, can in principle be done by all of the methods known to those skilled in the art. The introduction of the nucleic acid sequences gives rise to recombinant or transgenic organisms.

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In the case of microorganisms, those skilled in the art can find appropriate methods in the textbooks by Sambrook, J. et al. (1989) *Molecular cloning: A laboratory manual*, Cold Spring Harbor Laboratory Press, by F.M. Ausubel et al. (1994) *Current protocols in molecular biology*, John Wiley and Sons, by D.M. Glover et al., *DNA Cloning Vol.1*, (1995), IRL Press (ISBN 019-963476-9), by Kaiser et al. (1994) *Methods in Yeast Genetics*, Cold Spring Harbor Laboratory Press or Guthrie et al. *Guide to Yeast Genetics and Molecular Biology*, *Methods in Enzymology*, 1994, Academic Press.

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The transfer of foreign genes into the genome of a plant is called transformation. In doing this the methods described for the transformation and regeneration of plants from plant tissues or plant cells are utilized for transient or stable transformation. Suitable methods are protoplast transformation by poly(ethylene glycol)-induced DNA uptake, the „biolistic“ method using the gene cannon – referred to as the particle bombardment method, electroporation, the incubation of dry embryos in DNA solution, microinjection and gene transfer mediated by *Agrobacterium*. Said methods are described by way of example in B. Jenes et al., *Techniques for Gene Transfer*, in: *Transgenic Plants*, Vol. 1, Engineering and Utilization, eds. S.D. Kung and R. Wu, Academic Press (1993) 128-143 and in Potrykus *Annu. Rev. Plant Physiol. Plant Molec. Biol.* 42 (1991) 205-225). The nucleic acids or the construct to be expressed

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is preferably cloned into a vector which is suitable for transforming *Agrobacterium tumefaciens*, for example pBin19 (Bevan et al., Nucl. Acids Res. 12 (1984) 8711). *Agrobacteria* transformed by such a vector can then be used in known manner for the transformation of plants, in particular of crop plants such as by way of example tobacco plants, for example by bathing bruised leaves or chopped leaves in an agrobacterial solution and then culturing them in suitable media. The transformation of plants by means of *Agrobacterium tumefaciens* is described, for example, by Höfgen and Willmitzer in Nucl. Acid Res. (1988) 16, 9877 or is known inter alia from F.F. White, Vectors for Gene Transfer in Higher Plants; in Transgenic Plants, Vol. 1, Engineering and Utilization, eds. S.D. Kung and R. Wu, Academic Press, 1993, pp. 15-38.

Agrobacteria transformed by an expression vector according to the invention may likewise be used in known manner for the transformation of plants such as test plants like *Arabidopsis* or crop plants such as cereal crops, corn, oats, rye, barley, wheat, soybean, rice, cotton, sugar beet, canola, sunflower, flax, hemp, potatoes, tobacco, tomatoes, carrots, paprika, oilseed rape, tapioca, cassava, arrowroot, tagetes, alfalfa, lettuce and the various tree, nut and vine species, in particular of oil-containing crop plants such as soybean, peanut, castor oil plant, sunflower, corn, cotton, flax, oilseed rape, coconut, oil palm, safflower (*Carthamus tinctorius*) or cocoa bean, e.g. by bathing bruised leaves or chopped leaves in an agrobacterial solution and then culturing them in suitable media. For the production of PUFAs, for example stearidonic acid, eicosapentaenoic acid and docosahexaenoic acid, borage, linseed, sunflower, safflower or Primulaceae are advantageously suitable. Other suitable organisms for the production of for example γ -linoleic acid, dihomogamma-linoleic acid or arachidonic acid are for example linseed, sunflower or safflower.

The genetically modified plant cells may be regenerated by all of the methods known to those skilled in the art. Appropriate methods can be found in the publications referred to above by S.D. Kung and R. Wu, Potrykus or Höfgen and Willmitzer.

Accordingly, a further aspect of the invention relates to transgenic organisms transformed by at least one nucleic acid sequence, expression cassette or vector according to the invention as well as cells, cell cultures, tissue, parts – such as, for example,

leaves, roots, etc. in the case of plant organisms – or reproductive material derived from such organisms. The terms „host organism“, „host cell“, „recombinant (host) organism“ and „transgenic (host) cell“ are used here interchangeably. Of course these terms relate not only to the particular host organism or the particular target cell but also to the descendants or potential descendants of these organisms or cells. Since, due to mutation or environmental effects certain modifications may arise in successive generations, these descendants need not necessarily be identical with the parental cell but nevertheless are still encompassed by the term as used here.

For the purposes of the invention „transgenic“ or „recombinant“ means with regard for example to a nucleic acid sequence, an expression cassette (= gene construct, nucleic acid construct) or a vector containing the nucleic acid sequence according to the invention or an organism transformed by the nucleic acid sequences, expression cassette or vector according to the invention all those constructions produced by genetic engineering methods in which either

a) the nucleic acid sequence depicted in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9 or its derivatives or parts thereof or

b) a genetic control sequence functionally linked to the nucleic acid sequence described under (a), for example a 3'- and/or 5'- genetic control sequence such as a promoter or terminator, or

c) (a) and (b)

are not found in their natural, genetic environment or have been modified by genetic engineering methods, wherein the modification may by way of example be a substitution, addition, deletion, inversion or insertion of one or more nucleotide residues. Natural genetic environment means the natural genomic or chromosomal locus in the organism of origin or inside the host organism or presence in a genomic library. In the case of a genomic library the natural genetic environment of the nucleic acid sequence is preferably retained at least in part. The environment borders the nucleic acid sequence at least on one side and has a sequence length of at least 50 bp, preferably at least 500 bp, particularly preferably at least 1,000 bp, most particularly preferably at

least 5,000 bp. A naturally occurring expression cassette – for example the naturally occurring combination of the natural promoter of the nucleic acid sequence according to the invention with the corresponding Δ -8-desaturase, Δ -9-elongase and/or Δ -5-desaturase gene – turns into a transgenic expression cassette when the latter is modified by unnatural, synthetic („artificial“) methods such as by way of example a mutagenation. Appropriate methods are described by way of example in US 5,565,350 or WO 00/15815.

Suitable organisms or host organisms for the nucleic acid, expression cassette or vector according to the invention are advantageously in principle all organisms which are able to synthesize fatty acids, especially unsaturated fatty acids or are suitable for the expression of recombinant genes as described above. Further examples which may be mentioned are plants such as Arabidopsis, Asteraceae such as Calendula or crop plants such as soybean, peanut, castor oil plant, sunflower, corn, cotton, flax, oilseed rape, coconut, oil palm, safflower (Carthamus tinctorius) or cocoa bean, micro-organisms such as fungi, for example the genus Mortierella, Saprolegnia or Pythium, bacteria such as the genus Escherichia, yeasts such as the genus Saccharomyces, cyanobacteria, ciliates, algae or protozoa such as dinoflagellates like Cryptocodinium. Preference is given to organisms which can naturally synthesize oils in relatively large quantities such as fungi like Mortierella alpina, Pythium insidiosum or plants such as soybean, oilseed rape, coconut, oil palm, safflower, flax, castor oil plant, Calendula, peanut, cocoa bean or sunflower, or yeasts such as Saccharomyces cerevisiae and particular preference is given to soybean, flax, oilseed rape, sunflower, Calendula, Mortierella or Saccharomyces cerevisiae. In principle, apart from the transgenic organisms identified above, transgenic animals, advantageously nonhuman animals, are suitable, for example C. elegans.

Further useful host cells are identified in: Goeddel, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990).

Usable expression strains, e.g. those exhibiting a relatively low protease activity, are described in: Gottesman, S., *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California (1990) 119-128.

A further object of the invention relates to the use of an expression cassette containing DNA sequences encoding a Δ -8-desaturase, a Δ -9-elongase and/or a Δ -5-desaturase gene or DNA sequences hybridizing therewith for the transformation of plant cells, tissues or parts of plants. The aim of use is to increase the content of fatty acids, oils or lipids having an increased content of double bonds.

In doing so, depending on the choice of promoter, the Δ -8-desaturase, a Δ -9-elongase and/or a Δ -5-desaturase gene can be expressed specifically in the leaves, in the seeds, the nodules, in roots, in the stem or other parts of the plant. Those transgenic plants overproducing fatty acids, oils or lipids having at least three double bonds in the fatty acid molecule, the reproductive material thereof, together with the plant cells, tissues or parts thereof are a further object of the present invention.

The expression cassette or the nucleic acid sequences according to the invention containing a Δ -8-desaturase, a Δ -9-elongase and/or a Δ -5-desaturase gene sequence can, moreover, also be employed for the transformation of the organisms identified by way of example above such as bacteria, cyanobacteria, yeasts, filamentous fungi, ciliates and algae with the objective of increasing the content of fatty acids, oils or lipids possessing at least three double bonds.

Within the framework of the present invention, increasing the content of fatty acids, oils or lipids possessing at least three double bonds means, for example, the artificially acquired trait of increased biosynthetic performance due to functional overexpression of the Δ -8-desaturase, Δ -9-elongase and/or Δ -5-desaturase gene in the organisms according to the invention, advantageously in the transgenic plants according to the invention, by comparison with the nongenetically modified initial plants at least for the duration of at least one plant generation.

The preferred locus of biosynthesis, of fatty acids, oils or lipids for example, is generally the seed or cell layers of the seed so that a seed-specific expression of the Δ -8-desaturase, Δ -9-elongase and/or Δ -5-desaturase gene is appropriate. It is, however, obvious that the biosynthesis of fatty acids, oils or lipids need not be limited to the seed tissue but rather can also occur in tissue-specific manner in all other parts of the plant – in epidermis cells or in the nodules for example.

A constitutive expression of the exogenous Δ -8-desaturase, Δ -9-elongase and/or Δ -5-desaturase gene is, moreover, advantageous. On the other hand, however, an inducible expression may also appear desirable.

- 5 The efficiency of the expression of the Δ -8-desaturase, Δ -9-elongase and/or Δ -5-desaturase gene can be determined, for example, *in vitro* by shoot meristem propagation. In addition, an expression of the Δ -8-desaturase, Δ -9-elongase and/or Δ -5-desaturase gene modified in nature and level and its effect on fatty acid, oil or lipid biosynthesis performance can be tested on test plants in greenhouse trials.

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- An additional object of the invention comprises transgenic organisms such as transgenic plants transformed by an expression cassette containing a Δ -8-desaturase, a Δ -9-elongase and/or a Δ -5-desaturase gene sequence according to the invention or DNA sequences hybridizing therewith, as well as transgenic cells, tissue, parts and reproduction material of such plants. Particular preference is given in this case to transgenic crop plants such as by way of example barley, wheat, rye, oats, corn, soybean, rice, cotton, sugar beet, oilseed rape and canola, sunflower, flax, hemp, thistle, potatoes, tobacco, tomatoes, tapioca, cassava, arrowroot, alfalfa, lettuce and the various tree, nut and vine species.

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For the purposes of the invention plants are mono- and dicotyledonous plants, mosses or algae.

- A further refinement according to the invention are transgenic plants as described above which contain a nucleic acid sequence according to the invention or a expression cassette according to the invention.

Other objects of the invention are:

- 30 - A method for the transformation of a plant comprising the introduction of expression cassettes according to the invention containing a Δ -8-desaturase, a Δ -9-elongase and/or a Δ -5-desaturase gene sequence derived from algae such as Euglenia or Isochrysis, fungi such as Mortierella or mosses such as Physcomitrella or DNA' sequences hybridizing therewith into a plant cell, into callus tissue, an entire plant or protoplasts of plants.

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- A method for producing PUFAs, wherein the method comprises the growing of a transgenic organism comprising a nucleic acid as described herein or a vector encoding a Δ -8-desaturase, a Δ -9-elongase and/or a Δ -5-desaturase which specifically synthesize poly unsaturated fatty acids with at least three double bonds in the fatty acid molecule
 - Use of a Δ -8-desaturase, a Δ -9-elongase and/or a Δ -5-desaturase DNA gene sequence or DNA sequences hybridizing therewith for the production of plants having an increased content of fatty acids, oils or lipids having at least three double bonds due to the expression of said Δ -8-desaturase, Δ -9-elongase and/or Δ -5-desaturase DNA sequence in plants.
 - Proteins containing the amino acid sequences depicted in SEQ ID NO: 2, SEQ ID NO: 8 or its derivatives.
 - Use of said proteins having the sequences SEQ ID NO: 2 or SEQ ID NO: 8 for producing unsaturated fatty acids.
- A further object according to the invention is a method for producing unsaturated fatty acids comprising: introducing at least one said nucleic acid sequence described herein or at least one nucleic acid construct or vector containing said nucleic acid sequence into a preferably oil-producing organism such as a plant or a fungi; growing said organism; isolating oil contained in said organism; and liberating the fatty acids present in said oil. These unsaturated fatty acids advantageously contain at least three double bonds in the fatty acid molecule. The fatty acids may be liberated from the oils or lipids, for example by basic hydrolysis, e.g. using NaOH or KOH or by acid hydrolysis preferably in the presence of an alcohol such as methanol or ethanol. Said fatty acid liberation leads to free fatty acids or to the corresponding alkyl esters of the fatty acids.
- In principle an enzymatic hydrolysis for example with a lipase as enzyme is also possible. Starting from said free fatty acids or fatty acid alkyl esters mono-, di- and/or triglycerides can be synthesized either chemically or enzymatically. In another preferred embodiment of the inventive process the alkyl ester of the fatty acids are produced from the oils and lipids by transesterification with an enzyme or with conven-

tional chemistry. A preferred method is the production of the alkyl ester in the presence of alcoholates of the corresponding lower alcohols (C1 to C10 alcohols such as methanol, ethanol, propanol, butanol, hexanol etc.) such as methanolate or ethanolate. Therefore as the skilled worker knows the alcohol in the presence of a catalytic amount
5 of a base such as NaOH or KOH is added to the oils or lipids.

A method for producing triglycerides having an increased content of unsaturated fatty acids comprising: introducing at least one said nucleic acid sequence according to the invention or at least one expression cassette according to the invention into an
10 oil-producing organism; growing said organism; and isolating oil contained in said organism; is also numbered among the objects of the invention.

A further object according to the invention is a method for producing triglycerides having an increased content of unsaturated fatty acids by incubating triglycerides
15 containing saturated or unsaturated or saturated and unsaturated fatty acids with at least one of the proteins encoded by the sequences SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8 or SEQ ID NO: 10. The method is advantageously carried out in the presence of compounds which can take up or release reduction equivalents. The fatty acids can then be liberated from the triglycerides.

20 A further object according to the invention of said method for producing triglycerides having an increased content of unsaturated fatty acids advantageously having an increased content of unsaturated fatty acids is a method wherein the fatty acids are liberated from the triglycerides with the aid of basic hydrolysis known to those skilled
25 in the art or by means of an enzyme such as a lipase.

The methods specified above advantageously allow the synthesis of fatty acids or triglycerides having an increased content of fatty acids containing at least three double bonds in the fatty acid molecule.

30 The methods identified above advantageously allow the synthesis of fatty acids or triglycerides having an increased content of fatty acids containing at least three double bonds, wherein the substrate used for the reaction of the Δ -8-desaturase, Δ -9-elongase and/or Δ -5-desaturase is preferably - linoleic acid ($C_{20:2}^{\Delta 9,12}$) acid and/or

α -linolenic acid ($C_{18:2}^{\Delta 9,12,15}$). In this way the method identified above advantageously allows in particular the synthesis of fatty acids derived from linoleic acid ($C_{18:2}^{\Delta 9,12}$), α -linolenic acid ($C_{18:2}^{\Delta 9,12,15}$), γ -linoleic acid ($C_{18:3}^{\Delta 6,9,12}$), stearidonic acid ($C_{18:4}^{\Delta 6,9,12,15}$), dihomogamma-linoleic acid ($C_{20:3}^{\Delta 8,11,14}$) or such as by way of example eicosapentaenoic acid and arachidonic acid.

Examples of organisms for the said methods as described above are plants such as Arabidopsis, Primulaceae, borage, barley, wheat, rye, oats, corn, soybean, rice, cotton, sugar beet, oilseed rape and canola, sunflower, flax, hemp, potatoes, tobacco, tomatoes, rape, tapioca, cassava, arrowroot, alfalfa, peanut, castor oil plant, coconut, oil palm, safflower (*Carthamus tinctorius*) or cocoa bean, microorganisms such as the fungi *Mortierella*, *Saprolegnia* or *Pythium*, bacteria such as the genus *Escherichia*, cyanobacteria, yeasts such as the genus *Saccharomyces*, algae or protozoa such as dinoflagellates like *Cryptocodinium*. Preference is given to organisms which can naturally synthesize oils in relatively large quantities such as fungi like *Mortierella alpina*, *Pythium insidiosum* or plants such as soybean, oilseed rape, coconut, oil palm, safflower, castor oil plant, *Calendula*, peanut, cocoa bean or sunflower, or yeasts such as *Saccharomyces cerevisiae* and particular preference is given to soybean, oilseed rape, sunflower, flax, Primulaceae, borage, *Carthamus* or *Saccharomyces cerevisiae*.

Depending on the host organism, the organisms used in the methods are grown or cultured in the manner known to those skilled in the art. Microorganisms such as fungi or algae are usually grown in a liquid medium containing a carbon source, usually in the form of sugars, a nitrogen source, usually in the form of organic nitrogen sources such as yeast extract or salts such as ammonium sulfate, trace elements such as iron, manganese or magnesium salts and optionally vitamins at temperatures of between 10 °C and 60 °C, preferably between 15 °C and 40 °C with exposure to gaseous oxygen. In doing so the pH of the nutrient liquid may be kept at a fixed value, that is during growth it is or is not regulated. Growth can ensue in batch mode, semibatch mode or continuously. Nutrients can be provided at the start of fermentation or be fed in semicontinuously or continuously.

After transformation plants are first of all regenerated as described above and then cultured or cultivated as normal.

After growth the lipids are isolated from the organisms in the usual way. For this purpose, after harvesting the organisms may first of all be digested or used directly. The lipids are advantageously extracted using suitable solvents such as apolar solvents like hexane or ethanol, isopropanol or mixtures such as hexane/isopropanol, phenol/chloroform/isoamyl alcohol at temperatures of between 0 °C and 80 °C, preferably between 20 °C and 50 °C. The biomass is usually extracted with an excess of solvent, for example an excess of solvent to biomass of 1:4. The solvent is then removed, for example by distillation. Extraction can also be done using supercritical CO₂. After extraction the remaining biomass may be removed, for example by filtration.

The crude oil isolated in this way can then be further purified, for example by removing cloudiness by treatment with polar solvents such as acetone or chloroform and then filtration or centrifugation. Further purification through columns is also possible.

In order to obtain the free acids from the triglycerides the latter are saponified in the usual way.

A further object of the invention comprises unsaturated fatty acids and triglycerides having an increased content of unsaturated fatty acids produced by the methods identified above and use thereof for producing foods, animal feeds, cosmetics or pharmaceuticals. For this purpose the latter are added in customary quantities to the foods, the animal feed, the cosmetics or pharmaceuticals.

Said unsaturated fatty acids according to the invention as well as triglycerides having an increased content of unsaturated fatty acids produced by the methods identified above are the result of the expression of the nucleic acids according to the invention in the various host organisms. This results overall in a modification of the composition of the compounds in the host cell containing unsaturated fatty acids by comparison with the original starting host cells which do not contain the nucleic acids. These modifications are more marked in host organisms, for example plant cells, which naturally do not contain the proteins or enzymes encoded by the nucleic acids than in host organisms which naturally do contain the proteins or enzymes encoded by the nucleic acids. This gives rise to host organisms containing oils, lipids, phospholipids, sphingo-

lipids, glycolipids, triacylglycerols and/or free fatty acids having a higher content of PUFAs with at least three double bonds. For the purposes of the invention, by an increased content is meant that the host organisms contain at least 5 %, advantageously at least 10 %, preferably at least 20 %, particularly preferably at least 30 %, most particularly preferably at least 40 % more polyunsaturated fatty acids by comparison with the initial organism which does not contain the nucleic acids according to the invention. This is particularly the case for plants which do not naturally contain longer-chain polyunsaturated C₂₀ or C₂₂ fatty acids such as EPA or ARA. Due to the expression of the nucleic acids novel lipid compositions are produced by said means these being a further aspect of the invention.

The invention is explained in more detail by the following examples.

Examples

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Example 1: General cloning methods

The cloning methods, such as by way of example restriction cleavages, agarose gel electrophoresis, purification of DNA fragments, transfer of nucleic acids to nitro-cellulose and nylon membranes, linkage of DNA fragments, transformation of - Escherichia coli cells, culture of bacteria and sequence analysis of recombinant DNA, were carried out as described in Sambrook et al. (1989) (Cold Spring Harbor Laboratory Press: ISBN 0-87969-309-6).

25 Example 2: Sequence analysis of recombinant DNA

Sequencing of recombinant DNA molecules was done using a laser fluorescence DNA sequencer from the ABI company by the method of Sanger (Sanger et al. (1977) Proc. Natl. Acad. Sci. USA74, 5463-5467). Fragments resulting from a polymerase chain reaction were sequenced and checked to prevent polymerase errors in the constructs to be expressed.

Example 3: Cloning of the Δ -8-desaturase from *Euglena gracilis* (= SEQ ID NO: 1)

As a template for PCR amplification, cDNA from *Euglena gracilis* Strain Z was used.

The cDNA was synthesised from total RNA extracted from cultures of *E. gracilis*

5 strain Z. Unique primers to the initiating methionine and the stop codon of the *Euglena* Δ -8-desaturase were synthesized as shown, including restriction sites as detailed

Primer 1: EDELTA8BamF

10 ATGGATCCACCATGAAGTCAAAGCGCCAA

Primer 2: EDELTA8XhoR

ATCTCGAGTTATAGAGCCTTCCCCGC

15

PCR protocol

Addition temperature: 1 min at 45 °C

Denaturing temperature: 1 min at 94 °C

20 Elongation temperature: 2 min at 72 °C

Number of cycles: 30

The PCR products were separated on an agarose gel and a 1270 bp fragment was

isolated. The PCR fragment was cloned in the pGEM-T easy vector (Promega) and

25 the insert was then sequenced. This revealed the presence of an open reading

frame of 1266 base pairs, encoding a protein of 421 amino acid residues and a stop

codon. The C-terminus of the cloned Δ -8-desaturase has high homologies to the

Δ -8-desaturase published by Wallis and Browse (Archives of Biochem. and Biophysics, Vol. 365, No. 2, 1999) which is reported to be an enzyme of 422 residues; see also

30 related sequence by these authors [GenBank AF139720/ AAD45877] which purports to relate to the same Δ -8-desaturase but describes an open reading frame of 419

residues]. The deduced amino acid sequence the *Euglena* Δ -8-desaturase described

in this present invention differs from that previously described by heterogeneity at the

N-terminus. In particular, the first 25 amino acid residues of LARS Δ -8-desaturase is:

MKSKRQALP LTIDGTTYDVS AWWNF

Whereas the sequence described by Wallis & Browse is:

5

MKSKRQALS PLQLMEQTYDV SAWVN (as given in ABB 1999)

Or, alternatively

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MKSKRQALSPLQLMEQTYDVVNFH (as given in GenBank AAD45877)

Said heterogeneity present at the N-terminus of the desaturase sequence is not resultant of the PCR amplification or primers. The distinctions are true differences between the proteins.

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Example 4: Construction of transgenic plants expressing the *Isochrysis galbana* elongase component IgASE1

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The cloning of IgASE1 cDNA is described in: Qi, B., Beaudoin, F., Fraser, T., Stobart, A. K., Napier, J.A. and Lazarus, C.M. Identification of a cDNA encoding a novel C18-Δ-9-polyunsaturated fatty acid-specific elongating activity from the docosahexaenoic acid (DHA)-producing microalga, *Isochrysis galbana*. *FEBS Letters* 510, 159-165 (2002).

The cDNA was released from plasmid vector pCR2.1-TOPO by digestion with *KpnI*, and ligated into the *KpnI* site of the intermediate vector pBlueBac 4.5 (Invitrogen).

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Recombinant plasmids were screened for insert orientation with *EcoRI*. The insert was released from a selected plasmid with *PstI* plus *EcoRI* and ligated into binary vector plasmid pCB302-1 (Xiang *et al*, 1999) that had been cut with the same enzymes. This placed the IgASE1 coding region under the control of the CaMV 35S promoter as a translational fusion with the transit peptide of the small subunit of Rubisco (Xiang *et al*,

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1999), with the intention of targeting the elongase component to chloroplasts when expressed in transgenic plants. This recombinant binary vector was designated pCB302-1ASE. To construct a similar vector with expression of the elongase component targeted to the microsomal membrane, the IgASE1 coding region was removed from the intermediate vector by digestion with *BamHI* plus *SpeI*, and ligated into the

corresponding sites of pCB302-3 (Xiang *et al.*, 1999, in which the map of pCB302-3 is incorrect: the CaMV 35S promoter (plus omega' sequence) and nos terminator regions are reversed with respect to MCS2). This recombinant binary vector was designated pCB302-3ASE.

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Example 5: Plant expression of the elongase

Binary vectors were transferred to *Agrobacterium tumefaciens* strain GV3101 by electroporation; transformed colonies were selected on medium containing 50 µg ml⁻¹ kanamycin. Selected colonies were grown to stationary phase at 28°C, then the cells were concentrated by centrifugation and resuspended in a dipping solution containing 5% sucrose, 0.03% Silwet-177 and 10 mM MgCl₂.

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Seeds of *Arabidopsis thaliana* ecotype Columbia 4 were germinated on one-half-strength Murashige and Skoog medium, and seedlings were transferred to compost in 15 cm flower pots. Plants were grown to flowering stage in a growth cabinet at 21°C, with a 23 light and 1 hour dark cycle. Plant transformation was carried out by the floral dipping method of Clough and Bent (1998, Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant Journal* 16, 735-743 (1998), essentially as follows:

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For each construct two pots containing 16 plants were inverted in the dipping solutions containing transformed *A. tumefaciens* (described above). The plants were then covered with a plastic bag and left at room temperature in the dark overnight. The bag was then removed and the plants transferred to the growth cabinet. Dipping (with fresh *A. tumefaciens* solutions) was repeated after 5 days and the plants were allowed to set seed. Bulk seed from dipped plants (= T1 seed) was collected, and approximately 10000 seed sprinkled onto compost in a seed tray, and, after stratification at 4°C for 2 days, cultivated in the growth cabinet. When seedlings had reached the 2 to 4 true-leaf stage they were sprayed with Liberty herbicide (Aventis, 0.5g glufosinate-ammonium l⁻¹), and spraying was repeated one week later. Twelve herbicide-resistant plants were selected and potted on for each line (chloroplast or cytoplasm targeted elongase component), and allowed to self fertilize. Samples of T2 seed collected from these plants were germinated on one-half-strength Murashige and Skoog medium

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containing Liberty (5 mg glufosinate-ammonium l⁻¹). T3 seed collected from individual surviving plants was then again germinated on Liberty plates to screen for lines that had ceased segregating for herbicide resistance. Total fatty acids extracted from leaves of such lines were analysed and those with the greatest C20 content (CB12-4
5 with the chloroplast-targeted elongase component and CA1-9 with the cytoplasm-targeted elongase component) selected.

Example 6: Production of transgenic plants expressing the *Isochrysis galbana*
elongase component IgASE1 and the *Euglena gracilis* $\Delta 8$ desaturase
10 EUGD8

The $\Delta 8$ -desaturase coding region was removed from the yeast expression vector pESC-Trp with *Bam*HI plus *Xho*I, ligated into the *Bam*HI and *Xho*I sites of pBlueBac 4.5 (Invitrogen) and transformed into *E. coli* strain Tam1. The insert was removed
15 from a recombinant plasmid with *Bgl*II and *Bam*HI, ligated into the *Bam*HI site of pBECKS_{18,6} and transformed into *E. coli* strain Tam1. DNA minipreparations were made of the recombinant plasmids of 6 transformant colonies; these were digested with *Xho*I to determine the orientation of insertion of the desaturase coding region in the binary vector. One recombinant plasmid with the insert in the correct orientation for
20 expression from the CaMV 35S promoter was transferred to *Agrobacterium tumefaciens* strain GV3101 by electroporation and a dipping solution prepared from a transformed colony as described above.

Arabidopsis thaliana lines CB12-4 and CA1-9 (see above) were subjected to floral
25 dipping as described above. Approximately 2000 T1-seed from each line were spread on 15 cm petri dishes containing one-half-strength Murashige and Skoog (solid) medium supplemented with 50 μ g ml⁻¹ kanamycin and germinated in the growth cabinet. 12 kanamycin-resistant plants of the CA1-9 parental line and 3 plants of the CB12-4 parental line were transferred to potting compost and further cultivated in
30 the growth room. Fatty acid analysis was conducted on a leaf taken from each of the T2 plants, which were allowed to mature and set seed.

References

- McCormac, A.C., Elliott, M.C. and Chen, D-F.; pBECKS. A flexible series of binary vectors for *Agrobacterium*-mediated plant transformation. *Molecular Biotechnology* 8, 199-213 (1997).
- Xiang, C., Han, P., Lutziger, I., Wang, K. and Oliver, D.J.; A mini binary vector series for plant transformation. *Plant Molecular Biology* 40, 711-717 (1999).
- 10 Example 7: Production of transgenic plants expressing the *Isochrysis galbana* elongase component IgASE1 and the *Euglena gracilis* $\Delta 8$ desaturase EUGD8 and a $\Delta 5$ desaturase
- 15 The $\Delta 5$ desaturase from *Phaeodactylum tricornutum* was cloned into the pGPTV plasmid (Becker, D. et al.; Plant Mol. Biol. 20 (1992), 1195-1197) harbouring a hygromycin resistance selectable marker gene. For seed-specific expression the USP promoter from *Vicia faba* was cloned 5'-prime to the ATG of the $\Delta 5$ desaturase.
- 20 The binary vector was transferred to *Agrobacterium tumefaciens* strain GV 3101 and transformed colonies were selected on medium containing 30 μgml^{-1} hygromycin. Selected *Agrobacteria* were used for the transformation (flower transformation) of *Arabidopsis* plants carrying the T-DNA insertions with the $\Delta 9$ elongase and the $\Delta 5$ desaturase.
- 25 *Arabidopsis thaliana* seedlings were germinated on Murashige and Skoog medium containing hygromycin and resistant plants were transferred to the greenhouse.
- Seeds collected from individual plants were harvested and the total fatty acid profile
- 30 was analyzed using GC methods.

Example 8: Cloning of expression plasmids for seed-specific expression in plants

pBin-USP is a derivative of the plasmid pBin19. pBin-USP was produced from pBin19 by inserting a USP promoter as an EcoRI-BamHI fragment into pBin19 (Bevan et al. (1980) Nucl. Acids Res. 12, 8711). The polyadenylation signal is that of gene 3 of the T-DNA of the Ti plasmid pTiACH5 (Gielen et al., (1984) EMBO J. 3, 835), whereby nucleotides 11749-11939 were isolated as a PvuII-HindIII fragment and after addition of SphI linkers to the PvuII interface between the SphI-HindIII interface of the vector were cloned. The USP promoter corresponds to nucleotides 1-684 (gene bank accession number X56240), wherein a part of the nonencoding region of the USP gene is contained in the promoter. The promoter fragment running to 684 base pairs was amplified by standard methods by means of commercial T7 standard primer (Stratagene) and using a synthesized primer through a PCR reaction.

Primer sequence:

5'-GTCGACCCGCGGACTAGTGGGCCCTCTAGACCCGGGGGATCC
GGATCTGCTGGCTATGAA-3'

The PCR fragment was cut again using EcoRI/SalI and inserted into the vector pBin19 with OCS terminator. The plasmid having the designation pBinUSP was obtained. The constructs were used for transforming *Arabidopsis thaliana*, oilseed rape, tobacco and linseed.

Example 9: Production of transgenic oil crops

Production of transgenic plants (modified in accordance with Moloney et al., 1992, Plant Cell Reports, 8:238-242)

To produce transgenic oilseed rape plants binary vectors in *Agrobacterium tumefaciens* C58C1:pGV2260 or *Escherichia coli* were used (Deblaere et al, 1984, Nucl. Acids. Res. 13, 4777-4788). For transforming oilseed rape plants (var. Drakkar, NPZ Nordeutsche Pflanzenzucht, Hohenlieth, Germany) a 1:50 dilution of an overnight culture of a positively transformed agrobacteria colony in Murashige-Skoog medium

(Murashige and Skoog 1962 *Physiol. Plant.* 15, 473) containing 3 % of saccharose (3MS medium) was used. Petioles or hypocotyledons of freshly germinated sterile rape plants (approx. 1 cm² each) were incubated in a Petri dish with a 1:50 agrobacteria dilution for 5-10 minutes. This was followed by 3-day concubation in darkness at 25 °C on 3MS medium containing 0.8 % of Bacto-Agar. After three days, culturing was continued with 16 hours of light / 8 hours of darkness and in a weekly cycle on MS medium containing 500 mg/l of Claforan (sodium cefotaxime), 50 mg/l of kanamycin, 20 microM of benzylaminopurine (BAP) and 1.6 g/l of glucose. Growing shoots were transferred onto MS medium containing 2 % of saccharose, 250 mg/l of Claforan and 0.8 % of Bacto-Agar. If after three weeks no roots had formed 2-indolylbutyric acid was added to the medium as a growth hormone for rooting purposes.

Regenerated shoots were obtained on 2MS medium using kanamycin and Claforan, transferred into soil after rooting and after culturing grown for two weeks in a climate-controlled chamber, brought to blossom and after harvesting of ripe seed investigated for Δ -8-desaturase expression by means of lipid analyses. Lines having increased contents of double bonds at the Δ -8- position were identified. In the stably transformed transgenic lines functionally expressing the transgene it was found that there is an increased content of double bonds at the Δ -8-position by comparison with untransformed control plants.

The same procedure was done to create plants with Δ -9-elongase and/or Δ -5-desaturase activity.

a) Transgenic flax plants

Transgenic flax plants may be produced, for example by the by the method Bell et al., 1999, *In Vitro Cell. Dev. Biol.-Plant.* 35(6):456-465, by means of particle bombardment. Agrobacteria-mediated transformations can be produced, for example, as described by Mlynarova et al. (1994), *Plant Cell Report* 13: 282-285.

Example 10: Lipid extraction from seed and leave material

Plant material (approx 200 mg) was first of all mechanically homogenized by means of triturators in order to render it more amenable to extraction.

The disrupted cell sediment was hydrolyzed with 1 M methanolic hydrochloric acid and 5 % dimethoxypropane for 1h at 85 °C and the lipids were transmethylated. The resultant fatty acid methyl esters (FAMES) were extracted in hexane. The extracted

5 FAMES were analyzed by gas-liquid chromatograph using a capillary column (Chrom-pack, WCOT fused silica, CP wax 52 CB, 25 m, 0.32 mm) and a temperature gradient of from 170 °C to 240 °C in 20 min and 5 min at 240 °C. The identity of the fatty acid methyl esters was confirmed by comparison with corresponding FAME standards (Sigma). The identity and the position of the double bond was further analyzed by

10 means of GC-MS by suitable chemical derivatization of the FAME mixtures, e.g. to form 4,4-dimethoxyoxazoline derivatives (Christie, 1998).

Figure 1 shows the fatty acid profile (FAMES) of leaf tissue from wildtype *Arabidopsis thaliana* as a control. Figure 2 shows the fatty acid profile (FAMES) of leaf tissue from

15 transgenic *Arabidopsis* expressing the *Isochrysis* Δ -9-elongase (see example 4). This *Arabidopsis* line was subsequently re-transformed with the *Euglena* Δ -8-desaturase. The fatty acid profile (FAMES) of said double transformed *Arabidopsis* line (Line IsoElo X Eu D8 des) is given in Figure 3.

20 Furthermore this double transformed *Arabidopsis* line (Line IsoElo X Eu D8 des) was subsequently re-transformed with the *Mortierella* Δ 5 desaturase (Mort Δ 5) gene. The fatty acid profile (FAMES) of said triple transformed *Arabidopsis* line (Line IsoElo X EU D8 des x Mort Δ 5) is given in Figure 4.

25 Example 11: GC profiles of *Arabidopsis* leaf fatty acid methyl esters from different transgenics

Figure 5 shows GC profiles of *Arabidopsis* leaf fatty acid methyl esters extracted from wild type (WT 5a), single transgenic plants expressing *Isochrysis galbana* Δ 9 elongase

30 gene Ig ASE1 (5b), double transgenic plant expressing the Ig ASE1 and *Euglena* Δ 8 desaturase (EU Δ 8) genes (5c) and the triple transgenic plant expressing the Ig ASE1, Eu Δ 8 and the *Mortierella* Δ 5 desaturase (Mort Δ 5) genes (5d).

Table 1 shows the fatty acid composition of *Arabidopsis* plants prepared from wild type (Wt), single transgenic plant expressing the *Ischrysis galbana* IgASE1 elongase gene, double transgenic plants expressing the IgASE1 elongase gene and the *Euglena* $\Delta 8$ desaturase gene and triple transgenic plants expressing the IgASE1, the *Euglena* $\Delta 8$ and the *Mortierella* $\Delta 5$ desaturase gene. Analysis is of leaf tissue from rosette stage *Arabidopsis* plants. Each value represents the average of 2 measurements.

Fatty acid (mol% of total)	Plant source			
	Wt	IgASE1 transgenic	IgASE1+Eu $\Delta 8$ transgenic	IgASE1+Eu $\Delta 8$ +Mort $\Delta 5$ transgenic
16:0	19.9	19.2	14.7	14.2
16:1	2.8	3.3	1.8	2.3
16:3	13.1	12.2	19.9	15.4
18:0	1.7	2.4	0.8	1.5
18:1n-9	1.7	5.1	1.6	3.4
18:2n-6	11.2	9.0	4.2	6.6
18:3n-3	50.1	31.0	36.0	31.2
20:2n-6	-	7.9	0.9	3.2
20:3 $\Delta 5$,11,14	-			1.5
20:3n-6	-	-	9.1	1.5
20:4n-6 (ARA)	-	-		6.6
20:3n-3	-	9.9	4.0	4.8
20:4 $\Delta 5$,11,14,17	-	-	-	1.6
20:4n-3	-	-	7.2	2.9
20:5n-3 (EPA)	-	-	-	3.3
Total C20 PUFAs	-	17.8	21.2	22.2

All transgenes are under the control of the 35S-CaMV viral promoter. *Ischrysis* $\Delta 9$ elongase (IgASE1) with SSU Rubisco transit sequence [T-DNA Basta-r] were retransformed with *Euglena* $\Delta 8$ -desaturase^{mut175+313} [T-DNA Kanamycin-r]. The double transformed line, which is homozygous for both Basta-r and Kanamycin-r, were transformed again with *Mortierella* $\Delta 5$ desaturase (T-DNA Hygromycin-r). The resulting triple transformed line is homozygous for both Basta-r and Kanamycin-r, but heterozygous for Hygromycin-r.